The relationship between malaria and haemoglobin E in southern Vietnam

Thesis

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The Relationship Between Haemoglobin E and Malaria in Southern Vietnam

PhD thesis submitted on 15/09/06 by

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Abstract

Haemoglobin E is the most common single mutation haemoglobinopathy worldwide, presumably due to selective pressure from malaria. There is no good clinical data to support this hypothesis, however, and the laboratory data is conflicting. This thesis presents the results of prevalence studies, cross sectional association studies and a case control study undertaken to examine the relationship between haemoglobin E and malaria in southern Vietnam. The S'Tiếng ethnic group demonstrated a high prevalence of HbE and alpha thalassaemia, whilst the majority Kinh, and minorities from the north of Vietnam, had a low prevalence. The S'Tiếng also appeared to experience a higher burden of malaria infection, as determined by malaria parasite point prevalence and the age pattern of severe disease. There was no association between haemoglobin E and malaria in the cross sectional surveys once correction was made for ethnicity. A knowledge, attitudes and practice survey demonstrated a small difference in the proportion of S'Tiếng using bednets compared to other ethnic groups, but no other significant differences to account for their higher malaria burden. Malaria control programmes, which had been very successful elsewhere in Vietnam, have seemed slow to take effect in the central and southern highlands, particularly amongst ethnic minority groups. There appeared to be some success over the course of the study, however, with the result that recruitment to the case control study of severe malaria was slow, and there were insufficient cases from populations with high prevalences of haemoglobin E to draw any firm conclusions. The data gathered demonstrate a trend towards susceptibility to moderate and severe disease considered together, however, suggesting that any protective effect is likely to be modest.

Table of Contents

List of abbreviations

2,3-DPG	2,3-diphosphoglycerate
ARMS PCR	Amplification refractory mutation system PCR
DNA	Deoxyribonucleic acid
EC	European commission
EDTA	Ethylenediaminetetraacetic acid
EIR	Entomological inoculation rate
EM	electron microscopy
G6PD	Glucose-6-phosphate dehydrogenase
HbAE	Heterozygous haemoglobin E
Hb_{CS}	Haemoglobin Constant Spring
HbE	Haemoglobin E (similarly HbA, A2, C, S)
HbEE	Homozygous haemoglobin E
HCMC	Ho Chi Minh City
HDU	High dependency unit
HPLC	High performance liquid chromatography
HRP2	Histidine rich protein 2
HTD	Hospital for Tropical Diseases
HUVEC	Human umbilical vein endothelial cell
ICU	Intensive care unit
IFAT	Immunoflourescent antibody test
IMPE	The Institute of Malariology, Parasitology and Entomology, Ho Chi Minh City
ITN	Insecticide treated (bed)nets
KAP	Knowledge, attitudes and practice (study)
MCNV	Medische Comité Nederland-Vietnam
mRNA	Messenger RNA
NIMPE	The National Institute for Malariology, Parasitology and Entomology, Hanoi
OR	Odds ratio
PCR	Polymerase chain reaction
PICU	Paediatric intensive care unit
PRBC	Parasitised red blood cell
RBC	Red blood cell
RNA	Ribonucleic acid
TB	Tuberculosis
TDT	Transmission disequilibrium test
UNEP	United Nations Environment Programme
URTI	Upper respiratory tract infection
WHO	World Health Organisation
DTT	Dithiothreitol
YTTB	Y tế thôn bản (community health volunteer)
GDP	Gross domestic product

Table of figures

Declaration

This thesis reports the results from a series of sizeable clinical studies conducted predominantly in the community or district hospitals. Such studies always involve a large number of individuals in their execution. My role in this project was essentially one of design, supervision and analysis. I did not conduct any of the laboratory analyses, although did play a significant role in supervising the laboratory work conducted in Vietnam. A detailed breakdown of my contribution to the work described in this thesis follows, with acknowledgements. Almost every aspect of this project involved a degree of collaboration, or assistance and advice from others. Individuals making critical contributions to an aspect of the work I performed are listed in the detailed breakdown of my role in this project, otherwise the names of contributors are given in the acknowledgements, categorised by the role they played in the various studies herein described.

I declare that my role in the work described in this thesis was as follows:

I designed all the studies

Together with Jeremy Farrar, Dr Trần Tinh Hiền and Dr Nguyễn Hoan Phú, I negotiated the development of the study sites, and supervised their implementation

I designed the study forms

I supervised the implementation and conduct of all the studies, including: initial training; information and sample flows; data quality management, and troubleshooting. I performed these tasks through regular meetings with field workers, regular supervision meetings, and frequent discussions with team leaders

I wrote the databases for the cross sectional surveys, and the case control study, with some assistance in the latter from Hồ Vân Hiền

I supervised the data entry, and performed a significant proportion myself

Together with Dr Sarah Dunstan, I supervised the laboratory work undertaken in Vietnam

I performed an insignificant amount of the following work myself:

Haemoglobinopathy typing by HPLC, performed by Katie Miles, Angela Allen and Dr Chris Fisher in Oxford

Alpha thalassaemia genotyping by PCR, conducted by Katie Miles and Angela Allen in Oxford, and Nguyễn Thị Ngọc Quyền in Vietnam

DNA extraction and whole genome amplification

Reading of blood smears for malaria, performed by the HTD malaria laboratory team led by Bùi Thị Lý

Further details of the myriad individuals involved in this project are given in the acknowledgements below

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Case control study: Kasia Stepniewska
KAP: Dr Mary Chambers, Dr Lại Phùng Hưng

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All studies: Dr Trần Tinh Hiền, Dr Nguyễn Hoan Phú, Dr Đao Minh Triết, Dr Cao Quang Thái
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KAP: Dr Nguyễn Thế Khởi, Dr Lại Phùng Hưng
Case control study: Dr Đỗ Quang Anh, Dr Lê Thanh Long, Dr Lý Thanh Minh, Mai Văn Thống, Dr Phùng Đăng Cương, Dr Nguyễn Văn Chung

I performed very little of the laboratory analyses myself, as mentioned above. I am therefore indebted to the following dedicated individuals:

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Chapter 1 – Introduction

Introduction

The haemoglobinopathies, taken together, are the commonest single gene disorders in humans. The great JBS Haldane is usually credited with first postulating that this situation has evolved through selection for red cell disorders by malaria (Haldane 1949). The ensuing half century has seen the accumulation of compelling, though not yet conclusive, evidence in favour of this hypothesis. The relationship between the sickle cell gene and malaria is the most completely characterised, whilst that between malaria and glucose-6-phosphate dehydrogenase (G6PD) deficiency has been the most fiercely debated. The thalassaemias and haemoglobin E (HbE) have lagged behind in terms of both understanding and attention, despite being far more prevalent. Some evidence for the protective effect of alpha & beta thalassaemia has now been generated, although the mechanism remains unclear. To date there is no good quality clinical evidence, and conflicting laboratory data, on the relationship between HbE and malaria.

This PhD describes a programme of research aimed at documenting the existence and magnitude of the protective effect of HbE against malaria, and obtaining *in vivo* information on the possible mechanism of such protection. By way of introduction, this chapter describes the molecular basis, pathophysiology and clinical manifestations of haemoglobin E, highlights key points about the pathophysiology and epidemiology of malaria relevant to the studies undertaken, and discusses the evidence accumulated to date in support of the Haldane hypothesis, using sickle cell anaemia as a paradigm, and with particular reference to HbE.

Haemoglobin E

HbE was the 4th abnormal haemoglobin to be discovered, first reported by both Itano (Itano et al. 1954) and Chernoff (Chernoff et al. 1954) independently in 1954. The HbE

mutation lies at position 26 of the beta globin gene (Hunt et al. 1959), a G->A substitution resulting in glycine being replaced by lysine (Jonxis et al. 1956).

Biochemistry and molecular and cell biology

HbE migrates anodally of HbA and with HbC and HbA2 in routine electrophoresis on cellulose acetate at pH 8.4, and with HbA at pH 6.3 on citrate agar (Lachant 1987). It elutes with HbA2 in most cation exchange HPLC methods, including the commercially available Variant β short programme (Fucharoen et al. 1998; Mockenhaupt et al. 2004; Riou et al. 1997; Tan et al. 1993). Although E and A2 can be separated by isoelectric focusing (Bosisio et al. 1985), quantification of the HbA2/E band or fraction readily distinguishes HbE, as in heterozygotes it will comprise approximately 35% of total haemoglobin, and in homozygotes around 90%, whilst elevated HbA2 in β thalassaemia trait usually lies in the range of 3.5-7%, and rarely exceeds 8% (Weatherall et al. 2001a), p342). Heterozygous HbE erythrocytes are usually normochromic (Hinchliffe et al. 1995) and microcytic (Fairbanks et al. 1979; George et al. 1982), although this finding is not invariable (Bird et al. 1984; Chan et al. 2001; Marsh et al. 1983), whilst homozygous HbE cells are microcytic and often hypochromic (Fairbanks et al. 1979; Hurst et al. 1983; Marsh et al. 1983).

The haemoglobin E molecule is less stable than HbA. The isolated molecule precipitates at 50°C, and is more susceptible to acid denaturation (Yuthavong et al. 1975). Subsequent work suggests that HbE is stable at 37°C, but newly synthesized HbE appears unstable at 41°C in reticulocytes from homozygous HbE individuals, and at temperatures as low as 39°C in reticulocytes from HbE/β thalassaemic patients (Rees et al. 1998). Exacerbation of anaemia with intercurrent infections has not been described as part of homozygous HbE disease, so the relevance of the apparent thermal instability of HbE *in vivo* is uncertain.

The oxygen affinity of HbE was initially thought to be low, based on experiments on whole blood (Kolatat 1964) cited in (May et al. 1975), red cell preparations (Bellingham et al. 1968), and isolated haemoglobin (Thompson et al. 1965). Subsequent work on separated haemoglobin has shown a normal (Bunn et al. 1972; May et al. 1975) or even slightly raised (Gacon et al. 1974) oxygen affinity, with normal Bohr effect and interaction with 2,3-DPG. Heterozygous cells have consistently demonstrated a normal oxygen saturation curve (Bunn et al. 1972; Gacon et al. 1974; May et al. 1975), whilst that of homozygous cells does appear to be shifted to the right (Gacon et al. 1974; May et al. 1975). This latter effect appears to be due to elevated levels of 2,3-DPG in HbEE cells (Gacon et al. 1974; May et al. 1975), probably due to the associated anaemia.

HbE is more prone to oxidative attack than HbA (Frischer et al. 1975; Macdonald et al. 1983). The clinical significance of this is uncertain. An individual with *heterozygous* HbE who developed Heinz body anaemia in association with taking dapsone has been reported (Lachant et al. 1987b), but as the use of oxidant drugs, including dapsone and primaquine, is widespread in Southeast Asia, and no other cases have been reported, it seems unlikely that this represents a usual feature of HbE. On the other hand, effects of the oxidative instability have been demonstrated at the cellular level (Chiu et al. 1996; Frischer et al. 1975), and the protection afforded by HbE against malaria in one study was limited to those subjects regularly consuming fava beans, suggesting an oxidant mediated effect (Kitayaporn et al. 1992). The activity of anti-oxidant mechanisms in HbE red cells appears to be diminished, which might account for some of these phenomena (Lachant et al. 1987a).

HbE is inefficiently synthesised, with an apparent instability of β_E mRNA (Benz et al. 1981; Traeger et al. 1980). The discovery that the HbE mutation results not only in an abnormal β globin molecule, but also creates a novel mRNA splice site (Orkin et al. 1982) explained this surprising finding. Transcripts cleaved at this site are

non-functional, and it is estimated that only two thirds of β_E transcripts reach translation (Benz et al. 1981). The HbE mutation thus results in a mild β thalassaemia, as well as a structurally abnormal haemoglobin. Studies of globin chain imbalance in HbE containing cells have produced variable results. Imbalance is established in homozygous HbE erythrocytes, with α chain:non-α chain ratios of the order of 2 (Fairbanks et al. 1980; Wasi et al. 1982; Wong et al. 1982), but whilst some studies have found mild imbalance in heterozygous HbE erythrocytes (α:non-α ratios of 1.0-1.5) (Bird et al. 1984; Wong et al. 1982), others have not (α:non-α ratios of 0.75-1.0) (Wasi et al. 1982).

The relative contribution of the instability of HbE and the β thalassaemia element to the associated pathology is not completely clear. Membrane changes similar to those associated with β thalassaemia trait have been demonstrated in HbEE cells (Dorleac et al. 1984; Eastman et al. 1999), and deposits resembling agglomerates of precipitated alpha chains have been seen in homozygous, but not heterozygous, HbE cells in an electron microscopy study of erythroid precursors (Wickramasinghe et al. 1984). The pattern of abnormalities seen in HbEE erythroid precursors in this study were very similar to those seen in β thalassaemia trait. Red cell survival in homozygotes has been documented to be normal (Fairbanks et al. 1980), albeit in two individuals who were not anaemic, but mention of a slightly reduced lifespan is made by May and Huehns, without supplying data (May et al. 1975). A review of the pathophysiology of the thalassaemias can be found in Weatherall & Clegg. It is sufficient to note here that the imbalance in the quantity of alpha and beta globin chains produced is directly or indirectly responsible for the disease phenotype.

Clinical syndromes

The clinical phenotype of HbAE is asymptomatic and often clinically inapparent. Many heterozygous individuals will have a moderate microcytosis, usually with compensatory

polycythaemia (George et al. 1982). Anaemia is infrequent, and cases usually come to light on routine testing for another disease or in the investigation of the family of an individual with thalassaemia major. The clinical phenotype of HbEE is mild, and often asymptomatic. Microcytosis is usually marked, with compensatory polycythaemia. Mild anaemia is usual, but not invariable (Fairbanks et al. 1980; Hurst et al. 1983). Thus HbE alone does not result in significant disease. Many populations in which HbE is common, however, also have a relatively high prevalence of β thalassaemia. The clinical phenotype of the compound heterozygote HbE/β thalassaemia is extremely variable, ranging from asymptomatic to full blown thalassaemia major (Fucharoen et al. 1987b; Fucharoen et al. 2000; Weatherall et al. 2001e). The reasons for this variability are not entirely clear. The severity of the β thalassaemia mutation explains some, but not all, of the variation (Setianingsih et al. 1999), and individuals with mutations of similar severity can be affected differently (Nadkarni et al. 1999; Winichagoon et al. 2000). The list of potential candidate factors which do not seem to be important is long (eg (Wasi et al. 1985), although twin studies suggest that genetic factors are likely to account for much of the variation (Fucharoen et al. 1984). Investigations are ongoing into ancillary factors which might explain variations in some or all of the clinical picture (Weatherall et al. 2001e).

Haemoglobin E is usually found in much higher frequencies than β thalassaemia throughout its range. Despite its variable phenotype, HbE/β thalassaemia therefore accounts for at least half the cases of severe thalassaemia major in those countries in which HbE is prevalent (Brown et al. 1992; de Silva et al. 2000; Filon et al. 2000; George et al. 1992; Hao et al. 2001; Laosombat et al. 1992).

Distribution and population genetics

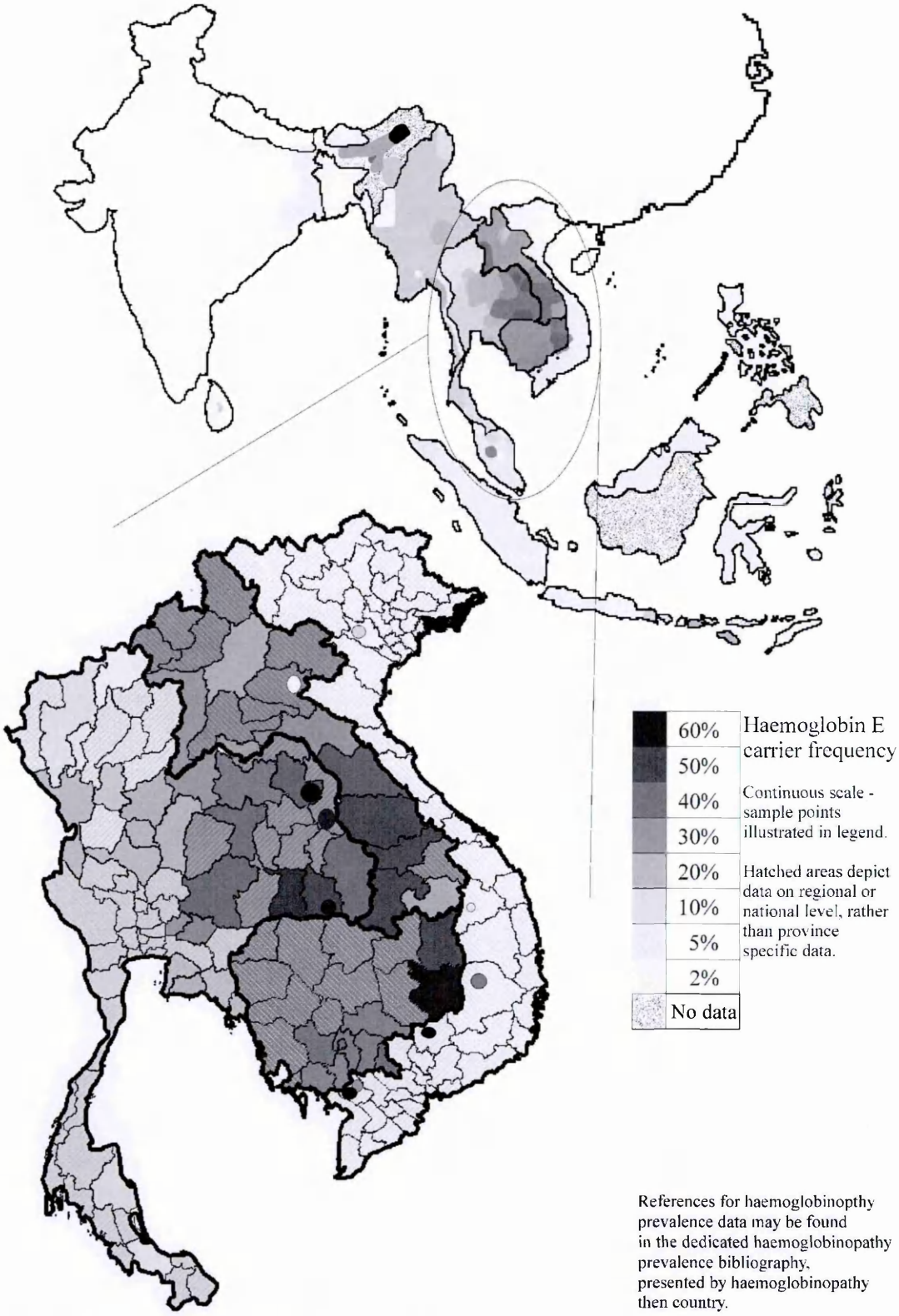
The extent of HbE in Southeast Asia became clear within a few years of its discovery, with reports from Cambodia (Brumpt et al. 1957), Indonesia (Eng et al. 1957; Lie-Injo

1955), Malaysia (Lehmann et al. 1956), Thailand (Na-Nakorn et al. 1956), and Burma (Colbourne et al. 1958), and slightly later reports from Laos (Baup 1964) and Vietnam (Anderson 1966). A survey amongst the Veddas, regarded as the original inhabitants of Sri Lanka, gave the first indication that HbE might be more widely distributed (Graff et al. 1955), followed by reports from Egypt (Hoerman et al. 1961) and Turkey (Aksoy 1960), neither currently considered high prevalence areas. The discovery of other, rare haemoglobin variants with similar electrophoretic properties as HbE (eg HbE Saskatoon) prompted a re-evaluation of the Southeast Asian situation by Blackwell and colleagues, who confirmed HbE in the Philippines (Blackwell et al. 1970), China (Blackwell et al. 1968), Sri Lanka (Blackwell et al. 1974), Thailand (Blackwell et al. 1975), Malaysia (Blackwell et al. 1971), Vietnam and Cambodia (Blackwell et al. 1972). Subsequent work has extended the distribution of HbE to Bangladesh and Assam in north east India (Das et al. 1971; Das et al. 1975; Das et al. 1991; Deka et al. 1987; Deka et al. 1988; Saha 1990). There have been occasional reports of HbE in Ghanaian (Yawson et al. 1973), West Indian (Bratt et al. 1991), black American (El-Shirbiny et al. 1980), Czech (Fortova et al. 1991) and other unspecified European families (Fairbanks et al. 1979). The first two were confirmed by detailed protein analysis, and the third family could trace recent Indian ancestry, but the other cases relied on electrophoresis and compatible haematological findings alone. 2 reports from the Congo (Gatti et al. 1968; Gatti et al. 1970) were not available for assessment.

Studies conducted in Malaysia in the late 1960's (Eng 1965; Lie-Injo et al. 1973), as well as a number of the Blackwell surveys cited above, focused attention on prevalence amongst ethnic minority groups. The extremely high frequencies of HbE among minority groups has been a recurrent motif in subsequent population based surveys (Das et al. 1991; De et al. 1997; Deka et al. 1988; Flatz et al. 2004; Fucharoen et al. 2002; Saha 1990; Sanguansermsri et al. 1987b; Sriboonlue et al. 1985), beginning with the classic work of Flatz and colleagues in Thailand (Flatz et al. 1965; Flatz 1967).

Although most of the groups in which HbE is prevalent are of putative Khmer origin, this is not true in Assam, where groups of Tibeto-Burmese origin predominate (Das et al. 1975; Das et al. 1985; Deka et al. 1988). The origin and spread of HbE have been the subject of much debate. Early haplotype studies in mainland Southeast Asian refugees found HbE on multiple haplotypes (Antonarakis et al. 1982), which has been confirmed in native Cambodian (Hundrieser et al. 1988d) and Thai (Fucharoen et al. 2002; Hundrieser et al. 1988d; Yongvanit et al. 1989) populations (the latter study including some ethnic minority groups closer to the Cambodians). It has been argued that this suggests multiple origins of the HbE gene (Antonarakis et al. 1982), which appeared to be confirmed by the discovery of a focus of high endemnicity among Tibeto-Burman groups in Assam with no apparent historical or ethnic relationship to the main focus of HbE (Deka et al. 1988). The E allele in one of these groups is carried on a Southeast Asian haplotype, however (Hundrieser et al. 1988a), which would appear more consistent with the argument that the probability of the HbE mutation occurring on more than one occasion is extremely small (Flint et al. 1998), and would not explain the current distribution of HbE very well (Livingstone 1989), so that subsequent gene conversion events are a more likely explanation for the haplotype diversity. The discordance between the predominant ethnic groupings affected in Assam and those in mainland Southeast Asia may reflect the danger of drawing conclusions about ethnic origins from current linguistic classifications, or the assimilation of small groups of migrants into dominant local cultures.

Three reviews summarise our current understanding of the distribution of Haemoglobin E in Southeast Asia (Fucharoen et al. 1987a), Northeast India (Deka et al. 1987), and Sri Lanka (de Silva et al. 2000). This data is summarised in Map 1.



Map 1: The distribution of Haemoglobin E.

The epicentre of HbE is the Cambodia/Laos/Thai border convergence, prevalence decreasing through Southeast Asia and northeast India with increasing distance from this point. Exceptions are the high frequency among certain ethnic minority groups in Assam, and the sharp drop in prevalence on reaching the Chinese peoples. The highest prevalences of HbE have been found amongst small ethnic minority groups, as noted

above, and there is a negative correlation between the frequencies of HbE and β thalassaemia in such groups. The majority populations of only three countries have a high prevalence of HbE, namely Cambodia, Laos and Thailand. These populations, presumably subject to greater mixing than minority groups, also have moderate or high prevalences of β thalassaemia, and thus suffer a heavy burden of HbE/β thalassaemia disease. It is thought that this situation has arisen through the mixing of "indigenous" peoples of Khmer origin with peoples descending from southern China (Fitzgerald 1972; Flatz 1967), but this hypothesis has been challenged (Poolsuwan 2003).

More recent migration continues to change the worldwide distribution of HbE. The Malayan origin of the Cape coloured population in South Africa presumably gives rise to the moderate frequency of HbE documented in Cape Town (Bird et al. 1982), and the episodes of politically driven migration from Southeast Asia are changing the pattern of thalassaemia in Europe and America (Choy et al. 2000; Dode et al. 1987), which will continue to alter with ongoing economically driven migration.

The mild phenotype of homozygous HbE, and essentially normal phenotype of heterozygous HbE, when considered with the negative correlation between HbE and β thalassaemia prevalence and the finding of extremely high prevalences of HbE only in small minority groups, raises the possibility that HbE might be considered an almost neutral mutation, elevated to high frequencies through the combination of strong founder effects and genetic drift in small populations. The widespread distribution of HbE indicates this is not the case, suggesting it must carry some selective advantage, but should we consider HbE as having any deleterious impact on evolutionary fitness in the absence of β thalassaemia? It is likely that anaemic homozygotes would fare less well in rigorous environments, although the negative selection pressure might not be great. A number of small studies have examined the effect of HbE on pregnancy outcome. Whilst an earlier study in India suggested HbE status had little adverse effect

on reproductive outcome (Deka 1981), a small Malaysian series suggested lower birth weight in babies born to mothers with HbE, but demonstrated no difference in fetal survival or prematurity (Ong 1975), and a subsequent study in India demonstrated increased fetal wastage in pregnant women with homozygous HbE compared to those with heterozygous HbE or HbA (Balgir 1992). No study suggested any difference in fertility, but menarche was slightly delayed in girls with HbE (Deka 1976), although the study design was weak. Thus HbE may well have a greater deleterious impact on fitness than would be expected from its clinical phenotype, mediated by its effects on reproduction.

The interaction with β thalassaemia is clearly an important consideration in the evolutionary impact of HbE, and the interaction with α thalassaemia might be expected to alter the red cell phenotype significantly by reducing the degree of globin chain imbalance. It is therefore appropriate to review, in brief, the genetics, clinical syndromes and distributions of other thalassaemias as part of this introduction. A discourse on the global epidemiology of the thalassaemias is beyond the scope of this thesis, and the interested reader is directed towards (Weatherall et al. 2001a), (Flint et al. 1998) and (Livingstone 1967). This review will focus on aspects relevant to the population genetics of the thalassaemia in mainland Southeast Asia, appropriate in view of the likely geographical origin of HbE.

Beta thalassaemia

Genetics

The beta globin gene complex lies on chromosome 11, and contains the genes for delta and gamma chains as well as beta globin itself. Nearly 200 mutations resulting in β thalassaemia have been characterised to date (http://globin.cse.psu.edu/globin/hbvar/), although only a few are common in any one region (Weatherall et al. 2001f). The majority are point mutations or small deletions of a few base pairs, and may be

classified as β^+ or β^0 depending on whether they impair or abolish beta chain production. Genetic factors which govern gamma chain expression and thus HbF levels are also important in determining the severity of the phenotype. Mutations which affect the beta globin promoter seem to allow upregulation of gamma and delta chain production, and may thus have a milder phenotype despite being β^0 mutations. Equally mutations carried on haplotypes with other features associated with increased HbF production will have a milder phenotype than that predicted on the basis of the associated beta chain production alone (Weatherall et al. 2001c).

Clinical syndromes

Heterozygous β thalassaemia is usually asymptomatic (apart from the very rare apparently dominantly inherited β thalassaemias). Mild anaemia and moderate microcytosis are usually evident (Weatherall et al. 2001d). An increase in HbA2 ($\alpha_2\delta_2$) is usually apparent, and forms the basis for the detection of β thalassaemia trait. Homozygous β thalassaemia (or, more usually, compound heterozygosity for two different β thalassaemia mutations) may result in two clinical syndromes of different severity: the majority will suffer thalassaemia major, but a minority will have thalassaemia intermedia. Thalassaemia major is a severe, transfusion dependent anaemia, with extensive extramedullary haematopoiesis resulting in bony weakness and deformities and hepatosplenomegaly. The children suffer iron overload (even in the absence of regular transfusion), infective and thrombotic susceptibilities, fail to thrive, and die in childhood without treatment (Weatherall et al. 2001d). Thalassaemia intermedia is poorly defined, but includes moderate anaemia that is not transfusion dependent and the absence of severe effects of extensive extramedullary haematopoiesis, although a degree of splenomegaly is common. Most children reach adulthood, but there have been no studies of longevity. Thalassaemia intermedia may result from homozygosity or compound heterozygosity for mild β^+ mutations (some of

which are common) or from beta globin mutations associated with increased HbF production, or through coinheritance of other mutations associated with raised HbF production or homozygous α^+ or heterozygous α^0 mutations (Weatherall et al. 2001c).

Distribution and population genetics

The severe phenotype of homozygous/compound heterozygous β thalassaemia is reflected in the much lower population prevalences of these mutations than can be found for HbE or α thalassaemia. A carrier frequency (for all β thalassaemia mutations other than HbE) of 6-10% may be regarded as high, and between 2 and 5% as moderate. Prevalences over 10% have been reported only from small, isolated population groups, and the highest published prevalence is 25%.

Data on the prevalence of β thalassaemia in mainland Southeast Asia are sparse compared to those for HbE. Only one report exists from Burma. Conducted in Rangoon, it suggests a prevalence of 4.5% in 112 Burmese. Published in the Journal of Burmese Life Sciences, the results are those quoted in Livingstone, so no methods or ethnic data were available. Most of the prevalence data for Cambodia, Laos & Vietnam come from studies conducted on refugees in France, the USA and Thailand. There is one good quality local study for Laos (Sicard et al. 1979), and 4 local studies in Vietnam. Of the latter, Bui Van Vien's thesis neglects to detail the HbA2 cut off used to diagnose β thalassaemia, and lists some suspiciously high prevalence figures, whilst the reports from Nguyen Cong Khanh and Bach Quoc Tuyen were not available for inspection, the results being quoted in Fucharoen and Winichagoon (Fucharoen et al. 1997). All these data are presented in table 1.1. The only good study from Malaysia suggests a prevalence of 2.2% in Malays (George et al. 1984). The data from Thailand are more plentiful and available, but are also problematic. The same data appears to have been published in a number of different places, falsely elevating the precision of the estimates, even when obvious duplicates have been removed. The much lower

overall prevalence of β thalassaemia necessitates larger studies to obtain good estimates, and increases the significance of small, in absolute terms, differences in prevalence. Rather than map the Thai data, therefore, they are presented in tables 1.2a & b.

Population group	Number of subjects	Carrier frequency	Reference
Cambodians			
Refugees in Thailand from rural areas in Cambodia. Mostly Khmer.	264	3.4	(Sanguansermsri et al. 1987a)
Refugees in Thailand from urban areas. Chinese admixture.	96	3.1	(Sanguansermsri et al. 1987a)
Cambodian exchange students in Paris	473	2.4	(Coquelet et al. 1983)
Khmer Refugees in San Francisco	59	3.4	(Hurst et al. 1983)
Laotians			
Laotians in Vientiane, Laos	494	6.4	(Sicard et al. 1979)
Mostly Lao (Lao Thai) resident in Ubol Rachathani, Thailand	438	2.5	(Sanguansermsri et al. 1987b)
Laotian exchange students in Paris	432	1.2	(Coquelet et al. 1983)
Refugees in Seattle	72	4.2	(Stein et al. 1984)
Refugees in San Francisco	116	5.1	(Hurst et al. 1983)
Lao Loum in the USA	46	4.0	(Monzon et al. 1986)
Vietnamese			
Kinh and unspecified Vietnamese (probably mostly Kinh)			
North Vietnamese	161	1.8	(De Traverse et al. 1959)
Vietnamese from the north in Saigon	153	3.9	(Le Xuan et al. 1968)
Kinh in Hanoi	401	1.5	Nguyen Cong Khanh*
Kinh in Hoa Binh (northern Vietnam)	605	5.3	(Bui 1999)
Kinh in northern Vietnam	512	1.2	Bach Quoc Tuyen*
Central Vietnamese	43	0	(De Traverse et al. 1959)
Vietnamese of central origin in Saigon	35	2.9	(Le Xuan et al. 1968)
South Vietnamese	255	1.69	(De Traverse et al. 1959)
Vietnamese from the south in Saigon	221	0.55	(Le Xuan et al. 1968)
Vietnamese refugees in Thailand	810	2.5	(Pornpatkul et al. 1980)
Vietnamese in Paris	188	2.2	(Coquelet et al. 1983)
Vietnamese refugees in Midwest US	58	2.0	(Monzon et al. 1986)
Vietnamese refugees in San Francisco	79	7.5	(Hurst et al. 1983)
Ethnic minorities			
Tai-Kadai language group			
Tay in north Vietnam	199	11	Nguyen Cong Khanh*
Viet-Muong language group			
Muong in Hoa Binh province	266	20	(Bui 1999)
Malayo-polynesian language group			
Ede, central Vietnam	371	1.3	Nguyen Cong Khanh*
Mon-Khmer language group			
Pak O, central Vietnam	228	8.3	Bach Quoc Tuyen*
Van Kieu, central Vietnam	78	2.6	Bach Quoc Tuyen*

Table 1.1: Beta thalassaemia prevalence data for Cambodia, Laos and Vietnam. Carrier frequencies are given as percentages. * quoted in (Fucharoen et al. 1987a)

Population group	Number of subjects	Carrier frequency	Reference
Northern Thailand			
Chiang Rai	32	6.3	(Flatz et al. 1965)
Chiang Mai			
Chieng Dao & Fang	110	4.5	
Doi Saked	159	4.4	
Chiang Mai city	119	9.2	(Flatz et al. 1965)
Pregnant women	131	3.8	
Sanpatong & Hod	219	5.5	
Lamphun	225	7.6	(Flatz et al. 1965)
Phitsanulok	2806	0.9	(Pravatmuang et al. 1995)
pregnant women	1015	1.5	(Pravatmuang et al. 1995)
	106	3.8	(Flatz et al. 1965)
Northeastern Thailand			
Udon Thani	315	5.0	(Wasi et al. 1967)
Khon Kaen	150	2.0	(Wasi et al. 1967)
Udon Thani & Khon Kaen provinces	88	5.7	(Flatz et al. 1965)
Ubon Ratchathani	130	2.3	(Flatz et al. 1965)
	565	3.0	(Wasi et al. 1967)
Nakhon Ratchasima	54	3.6	(Kruatrachue et al. 1969)
	180	1.6	(Flatz et al. 1965)
	54	3.7	(Kruatrachue et al. 1969)
Central Thailand			
Nakhon Sawan	93	2.2	(Flatz et al. 1965)
Nakhon Nayok	140	11.4	(Kruatrachue et al. 1969)
Urban Bangkok	149	2.7	(Flatz et al. 1965)
Rural Bangkok	121	1.7	(Flatz et al. 1965)
Phetchaburi	4426	2.8	(Pravatmuang et al. 1988)
Rayong	348	3.4	(Kruatrachue et al. 1969)

Table 1.2a: Beta thalassaemia amongst ethnic (and unspecified) Thai subjects. Carrier frequencies are given as percentages.

Population group	Number of subjects	Carrier frequency	Reference
Northern Thailand unless stated			
Tibeto-burman language group			
Lahu & Lisu	61	8.2	
Tai language group			
Thai Ya, Lue, Kuen	122	7.4	(Flatz et al. 1965)
Thai Yong in Lamphun	166	9.0	
Mostly Lao in Ubon Ratchathani, Northeastern Thailand	438	2.5	(Sanguansermsri et al. 1987b)
Tai Dam in Minnesota, USA	103	7.8	(Monzon et al. 1985)
Hmong			
Miao	62	9.7	
Mon-Khmer			
Northern Mon-Khmer			
Lawa	76	6.7	(Flatz et al. 1965)
Monic			
Mon	89	3.4	
Eastern Mon-Khmer			
Khmer in Surin province, Northeastern Thailand	133	0.8	
Mostly Khmer in Surin Prasat, Northeastern Thailand	299	3.3	(Sanguansermsri et al. 1987b)
Chinese			
Southern Chinese	66	6.1	(Flatz et al. 1965)

Table 1.2b: Beta thalassaemia prevalence amongst ethnic minorities in Thailand. Carrier frequencies are given as percentages.

Alpha thalassaemia

Genetics

The human genome contains 4 alpha globin genes, with two genes arranged sequentially within the alpha globin gene complex on chromosome 16. α thalassaemia mutations are denoted as α^+ if they result in the loss of function of one gene, and α^0 if they result in the loss of function of both genes. The majority of α thalassaemias are due to deletions (Higgs 1992). The two alpha globin genes are embedded within two highly homologous units. Reciprocal recombination between these units can result in α^+ mutations, in particular the $\alpha^{3.7}$ and $\alpha^{4.2}$ deletions (Higgs et al. 1989). $\alpha^{3.7}$ can be further divided into 3 subtypes depending on exactly where the recombination took place (Higgs et al. 1985), and none of these mutations has clearly defined breakpoints.

These α^+ deletions thus represent a family of mutations resulting from "legitimate" recombination events, and therefore arise much more frequently than the point mutations, microdeletions and illegitimate recombination events that account for the other α thalassaemias. Deletions which remove both alpha globin genes are predominantly found in Southeast Asia and the Mediterranean (Flint et al. 1993). Two such mutations are prevalent in mainland Southeast Asia, of which α^{SEA} is by far the most common (Liu et al. 1994; Winichagoon et al. 1984). Non-deletional α thalassaemia results, with one exception, in an α^+ phenotype, although the effects are usually more severe than deletional α^+ thalassaemia. This may be due to the majority of point mutations affecting the alpha 2 gene, which is usually dominant (responsible for 75% of alpha globin transcripts under normal conditions (Orkin et al. 1981)), but there also appears to be a lack of the compensation from the remaining alpha gene seen in deletional α^+ individuals (Weatherall et al. 2001g). The only non-deletional α thalassaemia mutation to reach polymorphic frequencies in mainland Southeast Asia is α^{CS}, a T->C mutation in codon 142 of the alpha 2 gene which results in the abolition of a stop codon, thus producing an elongated mRNA which is unstable. This mutation also results in an abnormal haemoglobin, $Hb_{ConstantSpring}$ (Hb_{CS}), and is associated with unique pathophysiological features (Schrier et al. 1997).

Clinical syndromes

Heterozygous α^+ thalassaemia is asymptomatic and often clinically inapparent, although a degree of microcytosis may result (Weatherall et al. 2001b). Homozygous α^+ and heterozygous α^0 thalassaemia are also asymptomatic, but microcytosis is a consistent feature. The disease states are HbH disease and HbBart's hydrops fetalis. The latter results from homozygosity (or compound heterozygosity) for α^0 mutations, and is incompatible with life, as there is no substitute for alpha chains in functional haemoglobin molecules (unlike β thalassaemia, where $\alpha_2\gamma_2$ and $\alpha_2\delta_2$ tetramers can

function). HbH disease results from compound heterozygosity for an α^0 and an α^+ mutation, or, rarely, from homozygosity or compound heterozygosity for severe non-deletional α thalassaemia mutations. It is often benign, resulting only in a mild degree of anaemia with marked microcytosis, but can be more debilitating, causing a chronic haemolytic anaemia with splenomegaly and occasional transfusion requirement. Individuals with HbH disease of any severity can be prone to haemolytic crises, usually precipitated by infection, characterised by a sudden drop in haemoglobin, often necessitating transfusion. Individuals with HbH disease are rarely transfusion dependent, although they may be prone to iron overload, and there is some suggestion of growth impairment in children (reviewed in Chui et al. 2003). There is no systematic data on life expectancy in HbH disease. Pregnancy outcome appears to be deleteriously affected by HbH disease, although no good systematic data exist (reviewed in Chui et al. 2003). Phenotypic variability in HbH disease is partially explained by genotype, with non-deletional HbH disease being more severe (Chen et al. 2000), but individuals with the same genotype can have differing clinical syndromes (Mirabile et al. 2000), implicating other factors.

Distribution and population genetics

The highest prevalences of α thalassaemia have, unsurprisingly, been reached in populations harbouring only α^+ alleles. Carrier frequencies of 90% or more have been recorded in some populations, such as the Tharu (Modiano et al. 1991) and Danuwar (Sakai et al. 2000) of Nepal. α^0 mutations are common in Southeast Asia, however, so the selective advantage is balanced by homozygote and compound heterozygote disadvantage. An exposition of the worldwide prevalence of α thalassaemia is beyond the scope of this thesis, and this section limits its range to mainland Southeast Asia. Most of the prevalence studies conducted in Southeast Asia have relied on measuring Hb Bart's in cord blood, which has been shown to miss a majority of single gene

deletions (Higgs et al. 1982). This limited data suggested a high prevalence (30-40%) in Laos (Sicard et al. 1979) and Northern Thailand (Fucharoen et al. 1987a), a moderate to high prevalence (15-25%) in central and southern Thailand (Fucharoen et al. 1987a), and a lower prevalence (5-10%) in northeastern Thailand (Fucharoen et al. 1987a) and Burma (Aung Than et al. 1971). Highly variable results have been reported from Malaysia, ranging from 5-10% (Lopez et al. 1971 and others cited in Fucharoen et al. 1987a), to nearly 50% (George et al. 1981). This unfeasibly high prevalence used 1% HbBart's as a cutoff, which may be too low . Molecular analysis of individuals with HbH disease (Winichagoon et al. 1984) has been helpful in quantifying the prevalences of different α^{+} mutations relative to one another (the $\alpha^{3.7}$ mutation being by far the commonest, followed by α^{CS}, with a few instances of $\alpha^{4.2}$) and of α^{0} deletions relative to one another (α^{SEA} constituting the vast majority, with occasional α^{THAI}), but cannot estimate the population prevalence of these mutations or the relative proportions of single to two gene deletions. This is clearly important, as populations with a high ratio of single:two gene deletions will be underestimated in Hb Bart's cord blood surveys. A few molecular population prevalence studies have been conducted in the region, which have generally demonstrated single:two gene deletion ratios of between 3:1 and 5:1. The paucity of data once again precludes useful mapping, and tables 1.3 and 1.4 show the distribution of α thalassaemia in mainland Southeast Asia as measured by genotypic surveys and HbBart's cord blood surveys respectively. Studies attempting to estimate the prevalence of α thalassaemia by excluding iron deficiency and beta globin anomalies in microcytic individuals are not presented. Table 1.5 shows the prevalence of Hb_{CS} in the region.

Mutation Population group	Number of subjects	Gene frequency	Reference
α_{SEA}			
Burma (Shan state)			
Pa O	407	0.010	
Bamar	311	0.003	(Than et al. 2005)
Shan	105	0.024	
Chinese	55	0.009	
Cambodia			
Cambodian refugees in Paris	114	0.018	(Dode et al. 1987)
Khmer refugees in Thailand	58	0.009	(Hundrieser et al. 1988b)
Thailand			
Northern Thai	106	0.024	(Hundrieser et al. 1988c)
Autochthonous rural dwellers in Chiang Mai ?Thai	215	0.070	(Lemmens-Zygulska et al. 1996)
Khon Kaen and Ubon Ratchathani	64	0.023	(Hundrieser et al. 1990)
Thai Bangkok	406	0.018[1]	Tanphaichitr 1985*
Thai Songhkla (southern Thailand)	301	0.022[1]	Sriroongrueng unpublished†
$\alpha_{3.7}$			
Burma (Shan state)			
Pa O	407	0.146	
Bamar	311	0.325	(Than et al. 2005)
Shan	105	0.162	
Chinese	55	0.045	
Cambodia			
Cambodian refugees in Paris	114	0.105	(Dode et al. 1987)
Khmer refugees in Thailand	58	0.155	(Hundrieser et al. 1988b)
Thailand			
Northern Thai	106	0.094	(Hundrieser et al. 1988c)
Autochthonous rural dwellers in Chiang Mai ?Thai	215	0.098	(Lemmens-Zygulska et al. 1996)
Khon Kaen and Ubon Ratchathani	64	0.148	(Hundrieser et al. 1990)
Thai Bangkok	406	0.083	Tanphaichitr 1985*
$\alpha_{4.2}$			
Burma (Shan state)			
Pa O	407	0	
Bamar	311	0.006	(Than et al. 2005)
Shan	105	0	
Chinese	55	0	
Thailand			
Northern Thai	106	0.005	(Hundrieser et al. 1988c)
Autochthonous rural dwellers in Chiang Mai ?Thai	215	0	(Lemmens-Zygulska et al. 1996)
Khon Kaen and Ubon Ratchathani	64	0.016	(Hundrieser et al. 1990)

Table 1.4: Alpha thalassaemia mutations in mainland Southeast Asia. [1]includes all 2 gene deletions, most of which will be α_{SEA}, [2]includes all single gene deletions, most of which will be $\alpha_{3.7}$, † cited in Fucharoen 1991 1289, * quoted in (Fucharoen et al. 1987a).

Population group	Number of subjects	Percentage with HbBart's	Reference
Burma			
Burmese	105	10.5	(Aung Than et al. 1971)
Laos			
Laotians in Vientiane	147	43.0	(Sicard et al. 1979)
Malaysia			
	162	1.2	(Vella 1962)
	100	58.0	(George et al. 1981)
Malays in Kuala Lumpur	344	7.3	(Lie-Injo et al. 1982)
	1431	6.9	(Lie-Injo 1973)
	1962	3.2	(Vella 1962)
Chinese in Kuala Lumpur	323	7.4	(Lie-Injo et al. 1982)
Chinese in Western Malaysia	568	6.9	(Lopez et al. 1971)
	222	0.9	(Vella 1962)
Indians in Kuala Lumpur	176	5.1	(Lie-Injo et al. 1982)
Thailand (all Thai)			
Bangkok	414	5.1	(Tuchinda et al. 1959)
	985	25.2	(Tanphaichitr et al. 1995)
Chiang Mai	287	30.7	(Na-Nakorn et al. 1970)
Khon Kaen	127	5.5	Saichua 1983*
Phetchaburi	4426	8.7	(Pravatmuang et al. 1988)
Vietnam			
Kinh in Hanoi	201	2.5	(Nguyen et al. 1992)
China			
Chinese (Hong Kong)	932	4.2	(Li et al. 1982)
	500	3.2	(Todd et al. 1969)

Table 1.3: Prevalence of HbBart's in cord blood in mainland Southeast Asia and China.

Population group	Number of subjects	Carrier frequency	Reference
Cambodia			
Urban Cambodian refugees	57	1.75	(Laig et al. 1990)
Laos			
Laotians in Vientiane	147	8.80	(Sicard et al. 1979)
Thailand			
Chiang Mai and Lamphun	151	6.51	(Laig et al. 1990)
Autochthonous rural dwellers in Chiang Mai ?Thai	215	2.31	(Lemmens-Zygulska et al. 1996)
Phitsanulok	2806	1.10	(Pravatmuang et al. 1995)
Khon Kaen and Ubon Ratchathani	64	10.64	(Hundrieser et al. 1990)
Phetchaburi	4426	0.40	(Pravatmuang et al. 1988)
Pregnant women in Bangkok	213	0.94	(Paritpokee et al. 2002)
Vietnam			
Kinh in Hoa Binh	605	0.34	(Bui 1999)
Muong in Hoa Binh	266	0.00	(Bui 1999)
Malaysia			
Malays			
Malays in Kuala Lumpur	344	1.16	(Lie-Injo et al. 1982)
Malaysians in Kuala Lumpur	1431	0.49	(Lie-Injo 1973)
Malays	536	2.24	(Lie-Injo et al. 1972)
Malay (Sumatran immigrants) Negri Sembilan Ulu Jempul / Kuala Pilah	629	2.38	(Ganesan et al. 1976)
Malay Ulu Trengganu Coastal	179	0.56	(Lie-Injo et al. 1971)
Malay Ulu Trengganu Inland	726	1.10	(Lie-Injo et al. 1971)
Malay men West Malaysia	916	1.64	(George et al. 1984)
Chinese			
Chinese in Kuala Lumpur	323	0.62	(Lie-Injo et al. 1982)
Chinese	607	0.66	(Lie-Injo et al. 1972)
Ethnic minorities			
Temuan Negri Sembilan	299	4.01	(Baer et al. 1976)
Temuan Ulu selangor	88	0.00	(Baer et al. 1976)
Temuan Selangor & Negri Sembilan	406	2.93	(Lie-Injo et al. 1973)

Table 1.5: Haemoglobin Constant Spring distribution in mainland Southeast Asia.

Malaria

Four species of *Plasmodium* cause disease in humans, of which two, *P. falciparum* and *P. vivax*, are prevalent in Southeast Asia. Worldwide, malaria is found in all tropical and many subtropical areas west of the 174th meridian, although historically malaria (in particular *P. vivax*) has been a public health problem in a number of temperate climates, including the Mediterranean, North America, and middle Europe, including Britain. Transmission is less frequent over 1500m above sea level, and rare above 2500m.

P. falciparum causes the majority of malaria infections worldwide, and with the exception of a few cases of severe anaemia attributed to *P. vivax*, is the only species causing severe malaria in humans.

Life cycle

Three of the four species of plasmodia infecting humans are specific for the human host (*P. malariae* is also capable of infecting non-human primates), and all are transmitted by female Anopheline mosquitoes. An infected mosquito carries *Plasmodium* sporozoites in her salivary glands. Mosquito saliva is injected into the victim on biting, acting as an anticoagulant and irritant to increase local blood flow. Sporozoites injected with the saliva are blood borne to the liver, where they invade hepatocytes and undergo a process of exoerythrocytic schizogony, producing thousands of merozoites, which are released into the blood stream and invade erythrocytes. *P. vivax* and *P. ovale* sporozoites pass through a hypnozoite stage. Whilst some of these hypnozoites go on to immediate schizont formation, others remain dormant for months or years. Reactivation of these hypnozoites causes late relapses. The time course of exoerythrocytic schizogony varies between species, from an average of 6 days in *falciparum* to 15 days in *malariae*. Once inside the erythrocyte, the merozoite develops through trophozoite stages to form a schizont, leading to membrane rupture and the release of further merozoites. The number of merozoites per schizont, and the periodicity of the erythrocytic schizogony cycle, differ between species. Some trophozoites differentiate into male and female gametocytes, which are not capable of further multiplication, but are ingested by a mosquito during a blood meal on an infective individual. Once inside the mosquito's stomach, the male gametocyte undergoes a remarkable physical transformation known as exflagellation, each flagellum being capable of fertilizing a female gametocyte. Once fertilised, the zygote transforms into a mobile oökinete, which migrates through the gut wall and takes up position on the extraluminal surface of the stomach, where it matures into an oöcyst, eventually containing hundreds of

sporozoites, which are released, on oöcyst rupture, into the haemolymph and carried to the salivary glands to begin the cycle again at the next blood meal. Mosquito host resistance to oökinete migration appears to be one of the major factors in determining whether a species can act as a malaria vector, and the reason why non-Anopheline species do not transmit malaria. The *Plasmodium* life cycle is depicted in fig 1.1. The duration of sporogeny varies both with temperature and species. *P. falciparum* and *P. vivax* have the shortest sporogenic cycle, at approximately 9 days at 28°C, and *P. malariae* the longest, averaging 15 days at the same temperature. As the ambient temperature drops towards 20°C, this duration lengthens almost exponentially, approaching 21 days for *P. falciparum* at 20°C. Development ceases entirely below 16°C.

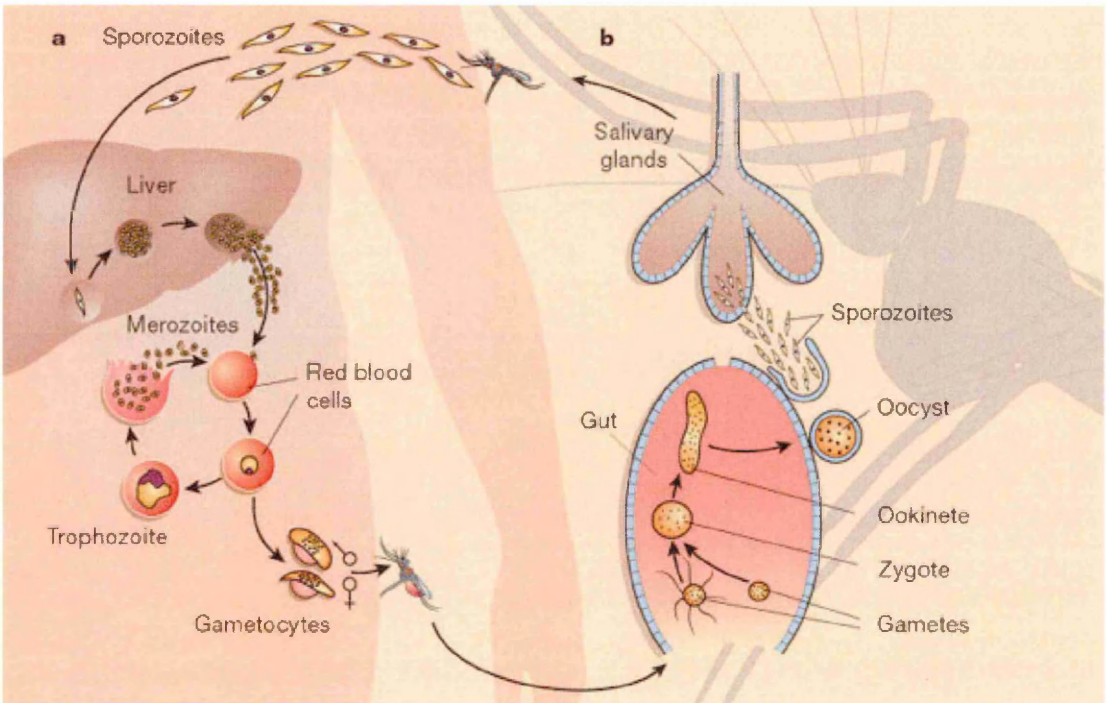


Fig 1.1. Life cycle of human *Plasmodium* parasites (from http://www.sanger.ac.uk/PostGenomics/plasmodium/presentations/plasmodium_lifecycle.shtm)

Vectorial capacity

Anopheline mosquitoes must bite twice, at least one sporogenic cycle apart, in order to transmit malaria. A number of factors may affect the likelihood that the cycle is completed. All anophelines will feed on humans or other animals, but differ in their

feeding preferences. Highly anthropophagic species are obviously more likely to take two successive meals from humans. Female anophelines only feed once per gonotrophic cycle (typically 48-72 hours, depending on ambient temperature), although this may require more than one bite if interrupted. Mosquito lifespan is another critical factor. High temperatures (>35°C) and low humidity (<50%) reduce lifespan, and there is significant variation between species. Average lifespan is 10-14 days, although some females may live up to 4 weeks. At lower ambient temperatures, the chance of the sexual stage of the *Plasmodium* life cycle completing before the mosquito host dies is small. Similarly at very high ambient temperatures or low humidity, the chance of a mosquito surviving between taking a meal of infected blood and becoming infective is low. Major vectors, therefore, not only have to be permissive to oökinete migration, but also be anthropophagic (*An.funestus, An.dirus*), long lived (*An.gambiae*) or breed in large quantities in close proximity to humans (*An.minimus*).

Natural history, clinical features & pathophysiology of malaria

Malariotherapy for syphilis has inadvertently provided us with excellent information on the natural history of malaria infections in naive individuals, although the use of partial treatment to modify the course of the disease obviously interferes with the "natural" history. Once the parasite has become apparent in the blood (limit of detection of light microscopy is 1-10 parasites/μl), there is a progressive rise in parasitaemia until this proliferative phase is terminated by treatment or the host immune response (presumably adaptive). This initial peak is the highest parasitaemia reached in the infection. In the absence of effective radical treatment there then follow a series of recrudescences, with progressively lower peak parasitaemias (Collins et al. 1999a). There may be a specific periodicity to these recurrences, and they can continue for at least a year, and possibly 2 or 3 (Eyles et al. 1951). This pattern may result from antigenic variation allowing the

parasite to periodically escape the attentions of the adaptive immune system (Hommel et al. 1983).

The patterns and mechanisms of antigenic variation amongst human malaria parasites has been most extensively investigated in *P. falciparum*. PfEMP1 appears to be one of the most important *P. falciparum* antigens. Expressed on the surface of parasitised red blood cells (PRBC's), PfEMP1 is involved in cytoadherence (Leech et al. 1984), one of the key pathogenic processes in *P. falciparum* malaria, and has been demonstrated to undergo variation *in vitro* (Biggs et al. 1991) and *in vivo* (Hommel et al. 1983; Staalsoe et al. 2002). PfEMP1 is under the control of the *var* gene family (Su et al. 1995). Less numerous than might be expected for genes coding for variant surface antigens, the mechanism and control of var gene switching remain incompletely understood. Multiple var genes appear to be transcribed, but not translated, during the ring stage of development, but only one is expressed during the trophozoite stage, at which point PfEMP1 appears on the PRBC surface (Chen et al. 1998). Control mechanisms may rely on surrounding genetic components (Horrocks et al. 2004) but do not involve genetic rearrangement (Scherf et al. 1998). Despite rapid var gene switching apparent in micromanipulation studies (Roberts et al. 1992), the same PfEMP1 appears to be expressed for many generations at the population level, unless exposed to selective pressures (Hommel et al. 1983; Staalsoe et al. 2002). New var genes are created during sexual reproduction, and, possibly, by mitotic recombination (by virtue of the telomeric location of most var gene families) (Freitas-Junior et al. 2000).

The interactions between variant surface antigens, the host immune system, and the resultant pattern of parasitaemia have also yet to be fully elucidated. The lower amplitude of the secondary peaks in parasitaemia is presumably due to immunity to unvarying minor strain specific antigens, or to activated cross-reactive immunity. It is equally unclear why the infection should eventually be cleared, well before exhausting

the full repertoire of variant surface antigens, but similar mechanisms may be at work. Only the initial peak of parasitaemia tends to be associated with symptoms (Collins et al. 1999a; Collins et al. 1999b), and this dissociation between infection and disease is a consistent feature of human plasmodial infections, giving rise, at its most extreme, to the phenomenon of asymptomatic parasitaemia. The timing of the appearance of gametocytes varies considerably between species and between individuals, and both triggers for and mechanisms of gametocytogenesis remain poorly understood. Gametocytes do not cause symptoms, as they neither sequester nor replicate, and the term "asymptomatic parasitaemia" always refers to the presence of asexual forms of *Plasmodium* in the peripheral blood.

Asymptomatic parasitaemia was observed by many early malaria researchers (Covell 1960). Initially a puzzle, we now understand that it can arise in one of three situations: asymptomatic recrudescence of a previous infection (whether terminated by drugs, the immune response, or a combination of both), asymptomatic infection in an individual with significant pre-immunity (such as most older children and adults in high transmission regions), or pre-symptomatic infection. It has been proposed that the last of these, not a truly asymptomatic state, accounts for most instances of "asymptomatic" parasitaemia in low transmission regions, at least for *P. falciparum* infections (Luxemburger et al. 1996), whereas the majority of patent infections in older children and adults in high transmission regions will be truly asymptomatic. The prevalence and degree of asymptomatic parasitaemia can be quite astonishing in such regions, where 80-90% of children can harbour parasites at any one time, and some children appear to tolerate very high parasitaemias without developing fever. This obviously creates considerably diagnostic difficulties for clinicians, and even more so for researchers attempting to attribute a febrile episode to malaria. Attempts to resolve this dilemma have focused on either the absence of an alternative cause for the fever, or generating age-related parasitaemia cut off values (Smith et al. 1994) (Luxemburger et al. 1996;

Rogier et al. 1996; Trape et al. 1987). The former is confounded by the wide variety of symptoms seen in malaria and creates practical difficulties due to the lack of basic diagnostic tools, such as auroscopes, in malarious areas, whilst the latter is further complicated by the apparent suppression of asymptomatic parasitaemia by intercurrent fever (Rooth et al. 1992), and has been criticised as an approach, though without realistic alternative suggestions (Bouvier et al. 1997).

Clinically apparent malarial disease is probably best classified into uncomplicated, complicated and severe. Uncomplicated malaria comprises fever, with or without non-specific symptoms such as headache, or constitutional symptoms, such as lethargy (but not to the point of prostration), rigors and chills. A number of other common symptoms, such as abdominal pain and mild diarrhoea, have been associated with malaria, making clinical diagnosis difficult (for example Luxemburger et al. 1998), particularly in the face of high prevalence of asymptomatic parasitaemia. The majority of clinically apparent malaria episodes fall into this category, including all those in individuals with a reasonable degree of pre-immunity. Severe malaria can be loosely defined as any clinical syndrome caused by malaria which is associated with an appreciable mortality. The difficulty in conducting research or formulating management algorithms around such a loose definition led to a consensus statement defining severe malaria in 1986, revised in 1990 Anonymous, (1990), details of which are given in the case control section of the methods chapter. Severe malaria is almost always caused by *P. falciparum*. Complicated malaria describes clinical episodes which entail more significant illness than uncomplicated disease but without reaching criteria for severe disease, and usually involve moderate impairment of a single organ/system (eg isolated mild jaundice, moderate anaemia, confusion or mild impairment of consciousness).

The pathophysiology of malaria is complicated, and different mechanisms would appear to be involved in different clinical syndromes (Mackintosh et al. 2004; Miller et al. 1994). Sequestration of PRBC's in capillary beds appears to be the most important pathogenic process in severe disease other than severe anaemia, and is unique to *P. falciparum*. An unproven axiom of malariology is that severe disease is associated with a high total body parasite burden. Host mechanisms which impair parasite multiplication, be they congenital (eg inherited alteration in erythrocytes) or acquired (eg pre-immunity), are associated with a reduced risk of severe malarial disease.

Immunity to malaria

The relationship between the malaria parasite and the human immune system is complicated and beyond the scope of this thesis. Appreciation of a few broad principles of immunity to malaria is important in understanding the complexity of malaria epidemiology, however, so these aspects, all of which concern the adaptive immune response to malaria (hereafter referred to as simply immunity, for the sake of brevity), are reviewed here. The remainder of this section concerns *P. falciparum* unless otherwise specified, as the immune response to other human malaria species has been much less extensively investigated.

Immunity to malaria is partial: individuals with immunity may still be infected, but develop fewer symptoms, and are much less likely to develop severe disease. Immunity may be thought of as developing in three stages: immunity to severe disease, anti-parasite immunity (limiting extent of parasitaemia and symptoms), and anti-infection immunity (dramatically reducing the chance that an infected bite will result in a patent parasitaemia). It is not clear if a single process, such as progressive acquisition of immunity to greater number of *P. falciparum* strains, is responsible, or whether different mechanisms underpin one or more of these stages of immunity. The very concept of a strain of *P. falciparum* is itself problematic: genetically identical sister parasites may

express different VSA's, and genetically distinct parasites might express the same PfEMP1. The ability of parasites to recrudesce in induced infections illustrates the importance of anti-VSA immunity in controlling parasitaemia, but the lack of symptoms usually associated with secondary peaks underlines the role of immunity against non-VSA antigens. The latter is also suggested by the failure of a previous infection to protect against heterologous challenge in induced malaria, the increase in the proportion of infections which are symptomatic at the beginning of the transmission season (Marsh 1992), although in both these situations the VSA's may also have changed, and, rather more strongly, by the association of symptoms with a rapid increase of genetically distinct parasites (Contamin et al. 1996). The discovery that *P. falciparum* isolates from children with severe disease were recognised more often, and at a younger age, amongst their contemporaries than isolates from children with mild disease (Bull et al. 1999), and that the VSA's expressed by these isolates corresponded to immunological "holes" in the affected children's repertoire, both underscores the importance of PfEMP1 in pathogenesis, and suggests that the acquisition of immunity to a limited number of PfEMP1's may be enough to abolish the risk of severe disease. It may not be necessary to recognise all severe disease associated PfEMP1's in order to be protected: the risk of non-cerebral severe malaria appeared to be negligible after as few as one or two clinical malaria episodes (Gupta et al. 1999). The concept that severe disease may be caused by parasites expressing a limited number of widely expressed PfEMP1's with certain specific "virulent" binding phenotypes (or the ability to bind multiple ligands (Heddini et al. 2001)), immunity to which, either by exposure or cross reactivity, can prevent the severe manifestations of *P. falciparum* infection, whilst anti-symptom and anti-infection immunity are mediated by the slower accretion of a greater repertoire of anti-parasite responses, is an attractive model (albeit one not completely supported by current data).Although the mechanism remains unclear, the clinical results are well documented: in high transmission regions the risk of severe disease peaks in young

children, and is effectively zero by the time they reach their teens. Asymptomatic parasitaemia is common throughout childhood, becoming less frequent in adulthood as the anti-infection levels of immunity are acquired. The pattern changes as transmission falls: fewer individuals will harbour parasites at any one time, more of those who are parasitaemic will be symptomatic, and severe disease occurs in a wider age range, including, in low transmission regions, adults and the elderly.

Immunity to malaria is not sustained. Alternatively expressed, continued exposure is necessary to maintain immunity. The time course of the decay in protection is uncertain, and will depend on the initial extent of pre-immunity, but individuals removed to a completely malaria free region may be at risk of developing severe disease within 10 years. The nature and frequency of exposure necessary to maintain disease specific immunity is uncertain.

There is no reliable assay for malaria immunity. Whilst levels of antibodies directed at *P. falciparum* surface antigens do correlate with protection in the ensuing few months, it has not been possible to predict a protective level of antibody. More recent work examining T cell responses to various antigens has given similar results.

The balance between partial but immeasurable immunity and a parasite with the potential for prolonged infection and recrudescence results in a dissociation between infection, parasitaemia, and disease, which causes significant difficulties in adequately measuring malaria.

Malaria epidemiology

A number of different classification systems have been used to describe the level of malaria transmission in a particular environment. The distinction between stable and unstable transmission is often made, referring to variations in degree of transmission over several years. Northern and eastern South Africa previously suffered the most

devastating pattern of unstable transmission, in which the absence of malaria for a number of years would be followed by moderate transmission for one or two years, followed by several more years without the vector. The effect of even moderate transmission on the background of a non-immune population is disastrous, however, with the vast majority of infections being symptomatic, and a relatively high proportion developing complications. Fortunately this situation is rare, but any area with unstable transmission is likely to experience a higher proportion of symptomatic cases during particularly malarious years than a region of stable transmission with comparable average transmission rates.

Seasonality is characteristic of most malarious areas, but varies in extent from the extremes of the Sahel, where three months of malaria are followed by nine months with no transmission, to the situation in most of tropical Southeast Asia, where transmission continues throughout the year but peaks during the rainy season. Only the former pattern is referred to as "seasonal".

The gold standard in assessing intensity of transmission is the entomological inoculation rate (EIR). This is difficult to measure, however, and impracticable for use in multiple areas simultaneously. Proxies in common use include the slide positive rate (SPR), an ill defined index which sometimes indicates the proportion of blood smears taken at healthcare facilities in a region which are positive, and sometimes describes the proportion of positive smears in a population based survey. It is poorly named, as it is rarely a rate, and suffers from the twin problems of asymptomatic parasitaemias and an extremely non-linear relationship with the EIR. The WHO classification based on the prevalence of splenomegaly in children between the ages of 2 and 9 inclusive is in common use, whereby a region is regarded as hypoendemic if the prevalence of splenomegaly is less than 10%, mesoendemic between 11 and 50%, hyperendemic consistently over 50%, with a 25% or more spleen rate in adults, and holoendemic if

consistently over 75% with a low spleen rate in adults. This system has the advantages of relying on an indicator of disease in an age group with, at best, partial immunity, of not requiring microscopy, and of consisting of only 4 categories. The last advantage can be disadvantageous in that it can conceal dramatic differences in EIR, particularly in high transmission regions, and thus being a poor index with which to compare adjacent areas, and of being misleading in environments in which other causes of splenomegaly are not rare, particularly if malaria transmission is low.

Haemoglobinopathies and malaria

Evidence for the protective effect of red cell disorders against malaria can be considered in four categories: macroepidemiological association between malaria endemic areas and haemoglobinopathies, microepidemiological correlation between the prevalence of malaria and that of haemoglobinopathies, *in vivo* evidence of protection (and possible mechanisms of protection) and *in vitro* evidence of impaired parasite growth in affected erythrocytes or an alternative mechanism of protection or both. The relationship between HbS and malaria remains the most completely characterised and will be used as an example before presenting the available data on the thalassaemias and HbE.

Haemoglobin S and malaria

Sickle cell anaemia was characterised as an autosomal recessive disorder of haemoglobin by 1952 (Beet 1949; Neel 1951), four decades after its initial description in 1910 (Herrick 1910), and 35 years after its familial nature was hinted at in a report of sickle cell disease in father and son in 1917 (Emmel 1917). The profound morphological changes induced in HbS containing cells by reducing agents encouraged the development of rapid screening tests, allowing early prevalence surveys in technologically inhospitable environments. Profound questions were raised by the appreciation of the extraordinarily high frequencies of such an obviously deleterious inherited condition in a large number of disparate populations. A number of hypotheses

were advanced, including increased fertility in HbAS women, but the ubiquitous and devastating nature of malaria, combined with the observation of a lesser degree of splenomegaly in individuals with HbS, led a number of researchers to postulate protection against malaria as the positive selective force behind HbS (Allison 1954a; Brain 1952).

The macroepidemiological evidence for this hypothesis was soon established, as the prevalence of HbS was documented in an increasing number of populations. The mutation was demonstrated to be common in many parts of sub-Saharan Africa (although virtually absent from southern Africa), the Middle East, the Indian sub-continent and certain regions of the Mediterranean (Flint et al. 1998; Livingstone 1985), all areas currently or previously endemic for malaria. An outstanding issue is the absence of HbS from other malarious areas. The origin of HbS is important in this regard: if the mutation arose once, and has spread by gene flow, then its current distribution is feasible. If, on the other hand, it has multiple origins, then the question of why it isn't prevalent in all malarious areas becomes more difficult to answer.

Two studies have examined the relationship between malaria and HbS at a microepidemiological level. Bienzle and colleagues examined the prevalence of HbS amongst two sets of individuals from the same ethnic group, one of which had remained close to their original mountain villages, where the estimated historical prevalence of malaria was approximately 8%, whilst the other had descended to live on the plains at the foot of the mountains, where the prevalence would have been closer to 50%. Twenty three percent of the latter group were HbS heterozygotes, compared to just 5% of the former (Bienzle et al. 1972). Allison compared the prevalence of HbS in geographically and ethnically defined groups exposed to either hyperendemic or seasonal/absent malaria transmission (Allison 1954b) and found that the former all demonstrated greater than 10% prevalence of HbS, whilst the latter all had <10% HbS.

The clinical evidence was beginning to accrue even before Haldane's expostulation. Beet (Beet 1946) observed that among hospital inpatients in a remote district of Rhodesia a higher percentage of those without HbS also carried malaria parasites than those with HbS (15.3 vs 9.8%), although the prevalence among patients admitted for malaria was similar to that amongst all inpatients. The difference in parasite prevalence is not significant, and the inpatients were drawn from disparate ethnic groups, with no comment on the ethnicity of parasitised compared to non-parasitised patients. It was noted that the difference in parasite prevalence was greater amongst cases admitted during the dry season (3.7 vs 11.4%) than during the wet season (16.6 vs 16.8%). The significance of these findings was not explored. The first reasonably rigorous clinical investigation of this hypothesis was carried out around Kampala in 1953 (Allison 1954a). Only 28% of children with sickle cell trait were parasitaemic compared to 46% of those with normal haemoglobin in a cross sectional survey of 100 under 5's of the Gandan ethnic group. Parasitaemia was also lower but was not adequately counted. Cross sectional surveys have rarely been fruitful in demonstrating protection by haemoglobinopathies, but HbS is an exception. Whilst few surveys have replicated the finding of lower prevalence in children with HbAS (Willcox et al. 1983a), and some have even demonstrated higher prevalences (Brabin et al. 2004; Ringelhann et al. 1976), these children appear to have significantly lower level parasitaemias (Boyo 1972; Willcox et al. 1983a). Several cohort studies have had similar findings (Fleming et al. 1979; Le Hesran et al. 1999; Stirnadel et al. 1999; Willcox et al. 1983a). The cohort studies pointed to an increasing effect of HbS with age. Willcox, Fleming and Stirnadel all found differences at all ages (over 6 months), but a more pronounced reduction in parasitaemia in AS children over the age of 1. A small Nigerian cohort found no differences in prevalence or parasitaemia in infants, whilst Le Hesran found no difference in infants, with increasing divergence in parasitaemias with age, reaching a marked difference over the age of 4 years.

Case control studies may be broadly divided into those that focus exclusively on severe malaria, and those that examine acute malaria cases. The latter usually defines a case population by presentation to a health care facility, and the expected severity of those cases will depend on the level of that facility. This is an important distinction.

The first evidence of protection against *severe* malaria was offered by Gilles in 1967. A rigorously defined group of 100 children with cerebral malaria were compared to a loosely defined control group of 200 children attending the clinic for reasons other than malaria. The prevalence of HbAS amongst the cases was 4%, compared to 18% among the controls (Gilles et al. 1967). A weakness of this study might be the requirement for a high parasite count, >100,000/μl, in order to enter the study. Given the available data on HbAS resulting in lower parasitaemias, and the inconsistent relationship between peripheral parasitaemia and disease, this might have biased the study. A similar criticism could be levelled at the findings of Martin and colleagues in Nigeria, who compared different groups of children presenting to hospital with convulsions. Amongst 30 with >100,000/μl only 1 had HbAS, compared to 8/38 with no apparent parasitaemia (Martin et al. 1979). Subsequent large case control studies in single ethnic groups, or using ethnically matched controls or stratifying for ethnic group, have proven the protective effect of HbS against severe disease (Hill et al. 1991; Modiano et al. 2001b). The protective effect is enormous, with odds ratios for HbAS children of the order of 0.1. These studies also examined protection against mild malaria, where a protective effect was also apparent, but of lower magnitude (OR's approximately 0.2-0.4). An effect of similar magnitude was demonstrated when all patients attending hospital with malaria were examined (Willcox et al. 1983b).

The increasing effect of HbS with increasing age might suggest an augmentation of immunity. The experimental infection of 30 Luo adults by Allison in 1954 suggested a more innate mechanism. Fifteen of these subjects had HbAS and 15 did not, they were

all apparently similar in malaria experience, and had lived outside an endemic area for 18 months. Only 2/15 with HbAS developed patent parasitaemia, compared with 14/15 with HbAA (Allison 1954a).

A possible mechanism for this direct effect was first suggested by Luzzatto in 1970, who observed that the rate of sickling was increased in parasitised red blood cells (PRBC's), and proposed that increased splenic clearance of sickled PRBC's might be the mechanism of protection (Luzzatto et al. 1970). The advent of a method for maintaining *P. falciparum* in continuous culture (Trager et al. 1978) created new opportunities to examine the relationship between erythrocytes and parasites in detail, although the lack of standardisation of conditions occasionally leads to difficulties in interpretation. A number of authors have demonstrated the normal growth and development of *P. falciparum* in AS and SS RBC's under optimum conditions (Ayi et al. 2004; Friedman 1978; Pasvol et al. 1978). If the oxygen tension is reduced to levels which might be found in deep capillary beds, however, multiplication is markedly impaired in AS cells, with inhibition of development suggested by the high proportion of abnormal trophozoites, whilst parasites in SS cells die (Friedman 1978; Pasvol et al. 1978). Both sets of authors observed that cells need not sickle in order for parasite development to be impaired, and suggested increased immune clearance, or impairment of parasite metabolism (eg due to HbS polymer being a poor substrate) as possible mechanisms. Some evidence for the latter was obtained by Orjih (Orjih 1999), who demonstrated reduced haemozoin formation in SS RBC's grown in low oxygen tension. This group confirmed normal *P. falciparum* multiplication in AS and SS RBC's under optimum conditions, but found only mild impairment in AS cells in low O_2 (and consequently did not show any significant metabolic impairment in such cells), whilst there was marked impairment in SS cells. The parasites used in these experiments had been passaged through AS cells for over 2 months in a low oxygen environment,

however, which might explain the relatively good growth rates, and suggests that *P. falciparum* can adapt to life inside AS cells.

Ring stage parasitised AS RBC's bound more IgG from autologous serum, had more evidence of oxidative damage, and were phagocytosed to a greater extent than ring stage parasitised normal RBC's (Ayi et al. 2004), providing direct evidence of an immune mediated effect. A number of authors have explored whether HbS might interfere with mechanisms of malaria pathogenesis, and whilst there appears to be no difference in rosetting between AS and AA PRBC's under normal conditions (Udomsangpetch et al. 1993b), deoxygenated AS RBC's rosetted less well than AA cells (Carlson et al. 1994). There is essentially no difference in cytoadherence between AA and AS PRBC's (Rowland et al. 1993).

There is thus macroepidemiological, microepidemiological and clinical evidence for the protective effect of HbS against malaria, with feasible mechanisms of protection demonstrated *in vitro* which are compatible with the *in vivo* data. There remain questions to be answered and inconsistencies to be ironed out, but overall the body of evidence is consistent and plausible. This is not always the case for other haemoglobinopathies.

Thalassaemias and malaria

A significant challenge to the Haldane hypothesis at the macroepidemiological level was the presence of α thalassaemia at moderate frequencies in non-malarious regions. Whilst β thalassaemia is only found in peoples from areas currently or previously endemic for malaria (Flint et al. 1998), Polynesian peoples carry α^+ thalassaemia at frequencies of up to 13%. Malaria is not and, as far as we know, has never been endemic in Polynesia, and there is little archaeological data to suggest the migration of the Polynesian peoples from malarious areas, although general theories about the population of the pacific islands propose they originated in Southeast Asia. The

categorisation of $\alpha^{3.7}$ mutations into $\alpha^{3.7}$I, $\alpha^{3.7}$II and $\alpha^{3.7}$III, and the demonstration that the last of these is found only in peoples from Polynesia and malaria endemic Melanesia (Hill et al. 1985) provided direct evidence of gene flow necessary to explain this inconsistency. $\alpha^{3.7}$ carries no discernable adverse effects in the absence of α^0 alleles, and it is not unfeasible that the mutation has been maintained in the population in the absence of any negative impact on fitness.

The earliest microepidemiological examination of the Haldane hypothesis for the thalassaemias was conducted in Sardinia (Siniscalco et al. 1966). Taking advantage of the variation in altitude of the island, the lack of population mobility and the historical isolation of the interior of the island, Siniscalco and colleagues showed a distinct negative gradient of β thalassaemia and G6PD deficiency from the malarious lowlands to the non-malarious highlands. One critique of Sinisalco's work (Brown 1981) highlights the difficulties inherent in all such microepidemiological studies, however thoroughly performed. Using data from extensive malaria surveys conducted in the 1930's and 1950's, Brown challenges the assumption of a direct relationship between altitude and malaria prevalence on the island. He also challenges the interpretation of the historical data on population movements, and proposes alternative explanations for the variation in prevalence of the red cell disorders based on gene flow into the lowland areas. All microepidemiological studies suffer from one or both of these weaknesses, as they are predicated on assumptions about historical reproductive patterns, population movements and malaria transmission. The evidence to confirm or refute these assumptions is even scantier for the majority of malarious areas than it is for Sardinia.

Most of the microepidemiological data for α thalassaemia come from Papua New Guinea, where malaria is hyperendemic in the lowlands, and virtually absent in the highlands. A high incidence of Hb Bart's in newborns in the lowlands, and its absence in the highlands, was reported by Oppenheimer (Oppenheimer et al. 1984), but the

number of highlander babies sampled was small. A much more extensive study by Flint and colleagues revealed a clear correlation between malaria endemnicity and α^+ thalassaemia prevalence (Flint et al. 1986), and similar data was presented by Yenchitsomanus (Yenchitsomanus et al. 1986). The former study included island Melanesia, and demonstrated a gradient of α^+ thalassaemia prevalence concordant with decreasing malaria prevalence from north to south and west to east. Some data has also been generated in Nepal, where the Tharu people, traditional inhabitants of the most malarious areas of the country, have an extremely high frequency of α^+ thalassaemia (Modiano et al. 1991). In another study 89% of the lowland dwelling Danuwar were shown to have α^+ thalassaemia, compared to 9% in the adjacent, highland dwelling Tamang (Sakai et al. 2000). These studies would have benefited from more detailed ethnological background information.

Compared to that for HbS, the clinical data for the protective effect of the thalassaemias against malaria is sparse and conflicting. In a cross sectional survey of 401 school and preschool children in Nigeria, there was no difference in prevalence of parasitaemia or median parasite density between normal and one or two gene deletion α thalassaemic individuals (Mockenhaupt et al. 1999). A subgroup of infants enrolled in an RCT of iron supplementation whose HbBart's had been measured at birth were bled at 6 and 12 months of age, and those with HbBart's tended to have a higher prevalence of parasitaemia than those without. Numbers were small, so the differences were not significant (Oppenheimer et al. 1987). In a cohort study of approx 250 children aged 3-8 in the Gambia no differences were observed in parasitaemia prevalence (in dry or rainy season surveys), malaria incidence, seropositivity, proliferative responses or interferon gamma production to a range of malaria antigens between children with normal alpha genes and those with single alpha gene deletion (Allen et al. 1993), although there was a suggestion that fewer of the latter exhibited splenomegaly during the dry season. A cohort study in Vanuatu found a higher spleen rate and a greater

incidence of *P. vivax* infections in young children with α^+ thalassaemia than those without, leading the authors to suggest that the protection of α thalassaemia against malaria mortality might be mediated by a "natural vaccination" effect (Williams et al. 1996).

A series of 6 cross sectional surveys through a population in Liberia showed children with β thalassaemia trait had lower parasitaemias in the 1-4 years age group, but similar prevalence (Willcox et al. 1983a). There was no difference in parasitaemia prevalence between children with β thalassaemia trait and normal children amongst 600 Thai under 3 year olds (Kruatrachue et al. 1969)(children with decreased osmotic fragility but normal A2 were not included as normal). These authors also found no differences in parasitaemia amongst this group or a further 82 children admitted to the district hospital with malaria. There was no difference in the incidence of severe disease or death amongst 204 children with or without β thalassaemia trait admitted with malaria (Kruatrachue et al. 1970).

The major evidence for the protective effect of alpha and beta thalassaemia against malaria comes from two case control studies. A study of 249 severe malaria cases in Papua New Guinea, individually matched for age, sex, and location to an equal number of community controls, showed an odds ratio (OR) for developing severe malaria of 0.4 for homozygous α^+ thalassaemia, and 0.6 for heterozygous α^+ thalassaemia, though the latter was not significant. Homozygous α^+ thalassaemia community controls exhibited a higher prevalence of *P. vivax* than normal controls (although heterozygous controls had a non-significantly lower prevalence of *P. vivax*). Prevalences of splenomegaly and *P. falciparum* were similar in all the community controls. Homozygous α^+ thalassaemia also protected against severe and mild non-malarial disease (OR 0.36, $p<0.001$ and OR 0.54, $p=0.036$ respectively), but not mild malaria (OR 0.57, $p=0.1$) (Allen et al. 1997). A case control study in Liberia of 558 patients attending hospital

with malaria, not necessarily severe, compared to population data from 1225 controls found only 5.5% of cases had β thalassaemia trait, compared to 9% of controls (OR 0.59, p=0.01) (Willcox et al. 1983b).

The *in vitro* evidence is similarly confused. Most studies have demonstrated normal parasite invasion (Bunyaratvej et al. 1992) and growth (Ayi et al. 2004; Friedman 1979; Kaminsky et al. 1986; Luzzi et al. 1990b; Senok et al. 1997a), although starting conditions and timing of endpoints varied widely between experiments. A number of variations on straightforward culture conditions have been attempted, based on the hypothesis that thalassaemia trait cells might support *P. falciparum* less well by virtue either of providing a reduced food source or of being more susceptible to oxidative attack. *P. falciparum* grew normally in microcytes from normal individuals with iron deficiency (Luzzi et al. 1990b), suggesting that microcytosis per se was irrelevant. Arguing that standard culture medium was overgenerous in its nutrient supply, Brockelman and colleagues found that parasite multiplication in β thalassaemia trait cells was impaired in a reduced medium (Brockelman et al. 1987). This medium contained less reduced glutathione as well as reduced amino acid content, however, making it impossible to distinguish nutritional from oxidant effects. A similar criticism might be levelled at the finding of impaired growth in β thalassaemia trait cells after 144 hours in culture without a change of medium (Kaminsky et al. 1986). Another group found divergence of multiplication curves in normal and thalassaemia trait erythrocytes after 72 hours of culture, even with daily changes of medium (Senok et al. 1997a). These results are difficult to interpret, given the differences between studies and the long duration of individual experiments potentially introducing additional unexplained factors. The second study also demonstrated a biologically implausible similarity of growth in α^0 and HbH cells. Senok and colleagues also explored whether varying susceptibilities among different subpopulations of erythrocytes might explain their drop in multiplication rate coincident with a third round of merozoite invasion. Cells of each

genotype were separated into 7 fractions on a ficoll gradient, with confirmation of increasing age with each fraction by RBC creatine measurement. The reticulocyte fraction was discarded. *P. falciparum* multiplication curves diverged from normal at 96 hours in the youngest fractions, and at 72 hours in the older fractions, in which were also noted many distorted schizonts which had failed to rupture. The addition of antioxidants improved parasite multiplication in the intermediate and old fractions (Senok et al. 1997b), leading the authors to postulate that the reduced oxidative defences of older erythrocytes were responsible for their greater antimalarial effect. Friedman and colleagues had already observed that high oxygen concentrations (30%) inhibited parasite multiplication to a greater extent in alpha & beta thalassaemia trait cells than in normal RBC's (Friedman 1979). Oxidising agents inhibited growth in beta, but not alpha, thalassaemia trait cells. Antioxidants reduced all these effects. Luzzi and colleagues, on the other hand, found no effect of oxygen or oxidising agents on parasite multiplication in α thalassaemia cells (Luzzi et al. 1991a).

The more abnormal, disease state thalassaemic erythrocytes do not support *P. falciparum* multiplication well (Bunyaratvej et al. 1992; Chotivanich et al. 2002; Ifediba et al. 1985; Yuthavong et al. 1987; Yuthavong et al. 1988). Occasional studies have not found significant differences between normal and HbH RBC's (Ayi et al. 2004; Senok et al. 1997a), unless, in one study, cultures were conducted in reduced medium (Brockelman et al. 1987). These results are not particularly interesting from an evolutionary standpoint, but provide evidence that inhibition of parasite multiplication is not a unique feature of HbS. Some of this effect in β thalassaemia cells may be due to the presence of HbF, which does not appear to affect invasion (Pasvol et al. 1977), but does impair development (Pasvol et al. 1976; Pasvol et al. 1977), probably by acting as a less digestible food source for the parasite (Shear et al. 1998).

The possibility of an immune mediated protective effect has been explored *in vitro*. Parasitised alpha, and, to a lesser extent, beta thalassaemia trait erythrocytes bound more IgG from pooled serum of individuals living in an endemic area than normal PRBC's (Luzzi et al. 1991b), and also bound more IgG from normal, unexposed serum (although at an order of magnitude less than the immune serum). Williams and colleagues confirmed this finding, and showed that it was not due to increased PfEMP1 expression (Williams et al. 2002). Ring stage parasitised beta trait RBC's bound more IgG from autologous serum, had more evidence of oxidative damage, and were phagocytosed to a greater extent than ring stage parasitised normal RBC's (although there was no difference once the trophozoite stage was reached). These findings were similar in HbH RBC's but not in homozygous α^+ erythrocytes (Ayi et al. 2004), which, in contrast to previous findings, did not bind more immune IgG. Luzzi had examined the change in IgG binding with parasite maturation, but did not report results separately for normal and thalassaemic cells, so these 2 sets of results are not necessary contradictory, but might indicate that β thalassaemia cells, binding more IgG earlier in the *Plasmodium* life cycle, might have a greater advantage than α thalassaemia, at least by this mechanism. Parasitised heterozygous α^+ and α^0 cells, hetero- and homozygous HbCS cells, HbH and HbH-CS cells were all phagocytosed more readily than normal cells, and this difference was greater for PRBC's, but with no clear differences between genotypes (Yuthavong et al. 1988).

Rosetting and cytoadherence of PRBC's have been associated with more severe disease. α^0 PRBC's showed normal adherence to C32 melanoma cells (Luzzi et al. 1990a), isolated CD36, ICAM-1 and thrombospondin (Williams et al. 2002), however heterozygous β thalassaemia and "heterozygous" α thalassaemia (unclear whether α^+ or α^0) cells were reported to have reduced adherence to HUVEC layers (Udomsangpetch et al. 1993a). α^{CS}, α^0 and β thalassaemia trait cells rosetted less than normal PRBC's in

competitive assays, and exhibited lower rosette disruption force (Carlson et al. 1994). This effect seemed to be predominantly due to microcytosis, although the phenomenon was not as pronounced in AA microcytes.

A Thai group found that some non-specific factor in the serum of unexposed individuals with thalassaemia disease states (HbH, HbH-CS or Beta/HbE) and negative IFAT's to crude *P. falciparum* and *P. vivax* extracts inhibited *P. falciparum* growth *in vitro*, particularly when added to cultures at the time of schizont rupture and merozoite reinvasion (Thanomsub et al. 1989). A direct toxic effect of the inhibition of heme polymerisation by the high levels of zinc protoporphyrin found in β thalassaemia trait erythrocytes has been postulated (Martiney et al. 1996). The same authors, however, demonstrated that exogenous ZnPP does not enter the parasite food vacuole, and thus has no effect on parasite growth, so this mechanism seems unlikely.

One paper reporting reduced parasite multiplication with morphologically abnormal maturation in alpha & beta thalassaemia RBC's, as well as reduced cytoadherence of these PRBC's to HUVEC layers, and reduced rosetting of PRBC's, is essentially uninterpretable as the genotypes of the cells are unclear (Udomsangpetch et al. 1993a). Although the thalassaemic cells are referred to as heterozygous, it is apparent from the text that the α thalassaemic cells are from compound heterozygotes with HbH disease, and the results would suggest that the beta thalassaemic cells are HbE/β thalassaemia compound heterozygotes.

In summary, two gene deletion α thalassaemia has been demonstrated to protect against severe malaria, but not mild malaria or parasitaemia, whilst α^+ trait has little documented effect. β thalassaemia trait protects against hospitalisation with malaria, but does not seem to affect prevalence or degree of parasitaemia. Alpha & beta thalassaemia trait erythrocytes appear to support *P. falciparum* growth and development normally, but may be more sensitive to oxidative stress, and seem to be better targeted

by the immune system than normal PRBC's. Thus whilst the macro and microepidemiological data are similar for the thalassaemias and HbS, the clinical data for the former is much less robust, consisting of a single, good quality case control study for each, and whilst reproducible mechanisms of protection consistent with the clinical data have been found *in vitro* for HbS, this is not the case for the thalassaemias.

Haemoglobin E and malaria

The distribution of haemoglobin E has been covered in detail above. All the areas in which HbE is polymorphic are currently malarious. Only one microepidemiological study has been published for HbE: the carrier frequency for HbE in individuals originating from hill villages in two provinces of northern Thailand was 12.3% compared to 8.2% in those from the rice plains ($p=0.036$). A more precise examination of the correlation between malaria prevalence and HbE was conducted in a third province, where the gene frequency of HbE in the malarious areas (average parasite prevalence 30%) was twice that in the lowland areas (average parasite prevalence 1%). Details of ethnicity were not given (Flatz et al. 1964).

The clinical data for HbE does not support an effect on mild or asymptomatic malaria, and whilst suggesting that it may reduce the severity of disease, there are no really good quality studies. No difference in overall or age specific prevalence was apparent in two cross sectional studies involving over 800 children in Thailand (Kruatrachue et al. 1961; Kruatrachue et al. 1969). HbE had no effect on parasitaemia in these children or those seen in local health centres or admitted to the district hospital. In the later, larger study children with decreased osmotic fragility but normal A2 were not included as normal in an attempt to exclude the effect of α thalassaemia. The same authors experimentally inoculated *P. vivax* into 3 AE adults and 6 AA adults, all of whom developed the same degree of parasitaemia with similar time courses (quoted in Kruatrachue et al. 1961).

No difference in the incidence of severe disease or death was apparent amongst 122 children with or without HbE admitted with malaria (Kruatrachue et al. 1970). This was a bit of a hit and miss case control study, however, as only a subset of the children had reasonable data collected on any one criterion for severity, only the cerebral subset is in any way consistent with current definitions of severe disease, and any child with any convulsion or impairment of consciousness was classified as severe for this purpose. A later and better Thai case control study compared 271 cases, between the ages of 2 and 60, presenting to Pong Nam Ron district hospital with fever and a positive blood smear for *P. falciparum* (mixed infections excluded), with controls drawn from patients with a negative blood smear attending for other reasons (eg anaemia, fever, jaundice, hepatomegaly or splenomegaly) which would have prompted investigation for malaria. Controls were frequency matched for sex and age band (2-15, 16-45, 46-60). HbE was not significantly protective by itself (87 HbAE & 13 HbEE amongst the cases vs 102 HbAE & 18 HbEE in the controls, p=0.082), but the interaction between eating fava beans and HbE genotype was significant in multiple logistic regression analysis, especially in those with higher parasitaemias. Consumption of fava beans by itself was protective (32 vs 51 p=0.023) (Kitayaporn et al. 1992). Whilst biologically plausible, given the apparent oxidative instability of HbE and the reduced anti-oxidant activity in HbE cells, the design of this study and the nature of the subgroup analysis cast some doubt over the validity of the result. A study amongst Burmese malaria inpatients found no difference between individuals with heterozygous HbE and those with normal haemoglobin in the proportion suffering severe disease (Oo et al. 1995), which contrasts with an opportunistic study performed at the Hospital for Tropical Diseases in Bangkok: probably the only reasonable clinical evidence for HbE protecting against malaria, this was a retrospective analysis of all patients admitted with *P. falciparum* malaria who had happened to have haemoglobin electrophoresis performed. Patients with β thalassaemia trait, G6PD deficiency or undetermined G6PD status, any other abnormal haemoglobin,

homozygous HbEE, and microcytosis in the absence of HbE were excluded. Ethnicity had been recorded, and analyses were stratified by ethnic group. In total there were 42 individuals with HbE and 175 without. Only one individual with HbE trait developed severe malaria, compared to 32 without (Hutagalung et al. 1999). Whilst this returns an impressive odds ratio, the strength of the effect is so out of keeping with other studies that, despite the best efforts of the authors to exclude confounders, certain aspects of the study design such as its retrospective nature, the selection of individuals for haemoglobin electrophoresis and the lack of information about the geographical origin of patients with regard to potential variation in malaria transmission raise concerns that this finding might be spurious.

The *in vitro* evidence is similarly conflicting. HbAE cells were invaded as efficiently as normal cells in a comparison of RBC deformability and ease of invasion by *P. falciparum*, although HbEE cells were invaded less well (Bunyaratvej et al. 1992). The relationship between reduced deformability and reduced invasion was not consistent. The invasion experiments were conducted at high haematocrit and with a high initial parasitaemia, so may not be comparable with other work. More recent invasion and growth work (Chotivanich et al. 2002) suggested that parasite multiplication was reduced to a similar extent in both AE and EE cells, and, in a set of elegant co-culture experiments, that AE and EE cells were more resistant to invasion than normal erythrocytes. Peculiarly HbEE cells were less resistant than AE cells which were on a par with Ebeta and HbH cells. The authors then went on to show that, uniquely amongst the cell types being examined, HbAE cells appeared to have resistant and susceptible subpopulations, as measured by the proportion of multiply infected RBC's. Two studies have found normal multiplication in both heterozygous and homozygous HbE cells (Santiyanont et al. 1981; Yuthavong et al. 1987), the latter group replicating this result in 30% oxygen. HbE cells supported parasite growth as well as normal cells in standard medium, but growth was reduced compared to normal

cells in a minimal medium containing 10% of the usual concentration of reduced glutathione and a 12% reduction in amino acid content (Brockelman et al. 1987). In contrast to these results, Nagel and colleagues found that multiplication was impaired in homozygous, but not heterozygous, HbE erythrocytes (Nagel et al. 1981), whilst Vernes and colleagues found poorer *P. falciparum* growth in AE cells even under optimum conditions (Vernes et al. 1986), with even more significant reduction in multiplication in EE cells. These differences were exacerbated when culture was conducted in 30% O2. Impairment of growth in high O2 was only partly ameliorated by the addition of vitamin C, and not at all by the addition of the reducing agent dithiothreitol. The differences between cell types were apparent under all conditions.

The phagocytosis of both unparasitised and parasitised HbE containing erythrocytes appears to be greater than HbAA cells. HbEE RBC's were taken up more readily than HbAE RBC's, and parasitised cells much more than unparasitised (Bunyaratvej et al. 1986).

In summary, the evidence for a protective effect of HbE against malaria is weaker even than that for the thalassaemias, similarly ridden with conflicting results, but without a single good quality clinical study to support the hypothesis. This body of work was aimed at rectifying this situation.

Plan of Thesis

Four chapters of this thesis follow a narrative line through the programme. Following the methods section (Chapter 2), a chapter is dedicated to the first cross sectional survey we performed, as it generated a number of hypotheses, as well as important baseline data, that shaped further studies. Chapter 4 presents the red cell disorder prevalences, and includes a detailed discussion of previous published data from the region. Chapter 5 describes the epidemiology of malaria in our study area, and includes discussion of associations between HbE and malaria indices discovered during the

course of the cross sectional surveys. Chapter 6 presents the behavioural survey under taken in October 2003. Chapter 7 presents the case control study. Chapter 8 draws together the conclusions from the different strands of the study, and discusses the need for further work, including the birth cohort study, which is currently ongoing.

Chapter 2 – Contexts and methods

Vietnam

Vegetation and terrain

Vietnam encompasses a wide variation in climate systems and terrain, generating a large number of different ecosystems. It stretches from the 9th to the 23rd parallel, a distance of over 1500 miles from most northerly to most southerly latitude. Bordered to the west by the Laotian plateau and the Annamite cordillera, and to the east by over 2000 miles of coastline, it is only 32 miles wide at its narrowest point. The southern bulb is dominated by the sprawling Mekong delta, whilst that in the north is divided between inhospitable highlands and the fertile Red River basin. War, development and agricultural encroachment have significantly diminished the ecological variety, abolishing some ecosystems entirely. Map 2 shows Vietnam in relief, with selected climatic data. The difficulties in using aggregated data of any kind are clear from a cursory inspection of this map: many of the central provinces span both coastal and mountain areas, make information at this administrative level difficult to interpret.

The majority of the studies were conducted in Phước Long district of Bình Phước province. Phước Long contains the southern foothills of the central Vietnamese plateau, which extend through into Bù Đăng and up through Tây Ninh into Đắc Lắc. Thác Mơ town, the capital of Phước Long, lies in the shadow of the unexpected Núi Bá Rà, which rises 500m above the surrounding countryside. Most of the province consists of low foothills, of up to 100m, rising to 200m in Phước Long, and 400m in Bù Đăng. According to 1997 data, approximately 33% of cultivated land is under cashew, 41% rubber, 13% paddy, 7% other food cereals including cassava, 4% coffee and 3% other cash crops including pepper (http://www.osh.netnam.vn/html/THONGTIN/61TINH/Binhph~1.htm). Fig 2.1 comprises pictures of typical terrain and vegetation. Most of the province was densely forested, and formed the southern end of the Hồ Chí

Minh trail during the "American War"[†] . The deforestation caused by napalm and agent orange has been exacerbated by land clearance for agriculture by immigrants and the displaced ethnic minorities. Away from cultivated areas, and predominantly towards the Cambodian border, secondary forests dominated by bamboo with a few persistent emergents may be found. Very little primary forest remains, and even protected forest areas are being rapidly destroyed (the Bù Gia Phúc protected area alone was estimated to have lost 85% of its forest cover by late 2004). There are major lakes in Phước Long and Bù Đăng, including a large reservoir created by the dam built in 1980 to feed Thác Mơ hydroelectric power station. The region is crossed by 3 recognised rivers and many small rocky tributaries.

Population

Vietnam is a country of 80 million people, comprising a majority 85% Kinh (also known as Vietnamese or Việt), and 54 minority groups contributing between 10,000 and 2.5 million individuals to the remaining 15% (1999 census). There is a strong sense of ethnic identity amongst both majority and minority groups, and, with a few exceptions, intermarriage is rare. The population structure is still rural-developing, with 70-80% of the population living in rural areas (various sources), and 33% under the age of 15 (1999 census). Vietnam has a one couple – two children policy, which is adhered to by a significant proportion of urban dwellers, and that section of rural society attached to the authorities. It is generally ignored by peasant farmers and, in particular, by most ethnic minority families. Population growth rate has slowed significantly to 1.4% in 2001 (General Statistics Office, Vietnam – available online at http://www.gso.gov.vn/default_en.aspx? tabid=467&idmid=3&ItemID=3322), however this headline figure probably conceals great differences between the urban elite at one extreme, and the rural ethnic minority poor at the other. There have been significant,

[†] The Vietnam War is known in Vietnam as the American war, to distinguish it from the French War, the Chinese War (parts 1-6) etc.

orchestrated population movements over the last 30 years, partly for political reasons, and, after 1975, partly due to the inability of the land in the north to support its population, whilst certain areas of the south were sparsely populated ((Evans 1992), also reasonable brief summary at hrw.org/reports/2002/vietnam/viet0402-06.htm). Migration continues, although in a slightly less regimented fashion. Certain provinces have been designated "acceptor provinces" due to their relatively low population density, and two of these are the most malarious provinces of Bình Phước and Đắc Lắc.

Ethnography[§]

The Kinh are thought to have originated in the Red River basin around the region of present day Hanoi, spread through the north of Vietnam during the first millennium AD, continued the push south since the 16th century, and, eventually, with the defeat and disappearance of the Cham empire in southern central Vietnam, extended throughout the country by the time of the great Nguyen Dynasties in the 17th and 18th centuries. In general terms this expansion has followed the lowland areas suitable for paddy farming, and forays into the highlands have been predominantly to trade(Andrew Hardy, personal communication). It is only over the past century that the Kinh have settled in the higher lands in significant numbers.

The S'tiếng (occasionally written X'Tieng) and M'Nông fall into the Mon-Khmer language group. The S'tiếng are a relatively small ethnic group (table 2.1) with a strong ethnic identity and a reputation for fierce independence. Their historical territory was densely forested as recently as 150 years ago, extending north into present day Cambodia and south to Thù Dầu Một, the current capital of Bình Dương province. Their range is now much more limited, however. The M'Nông are a larger ethnic group with subdivisions that vary in some details of agricultural practice and reproductive

[§] The information presented is a synthesis from 5 main sources listed separately at the end of the bibliography under "Ethnology references". It is difficult to attribute any one piece of data to any one source.

behaviour. Their historical territory is further north than that of the S'tiếng, in the southern central highlands in the modern day provinces of Đắc Lắc and Kontum.

The Tày and Nùng are the largest ethnic minority groups in Vietnam, but are recent immigrants to the south, where they are present in much smaller numbers than in the north. They are very closely related to one another, and to the Choang group of Guangxi and Guangdong provinces in southern China. The common ancestor of these 3 minority groups, the Tày Au, were at one stage the dominant military and political force in the North of Vietnam, briefly subjugating the Kinh Vietnamese to form the kingdom of Au Lac.

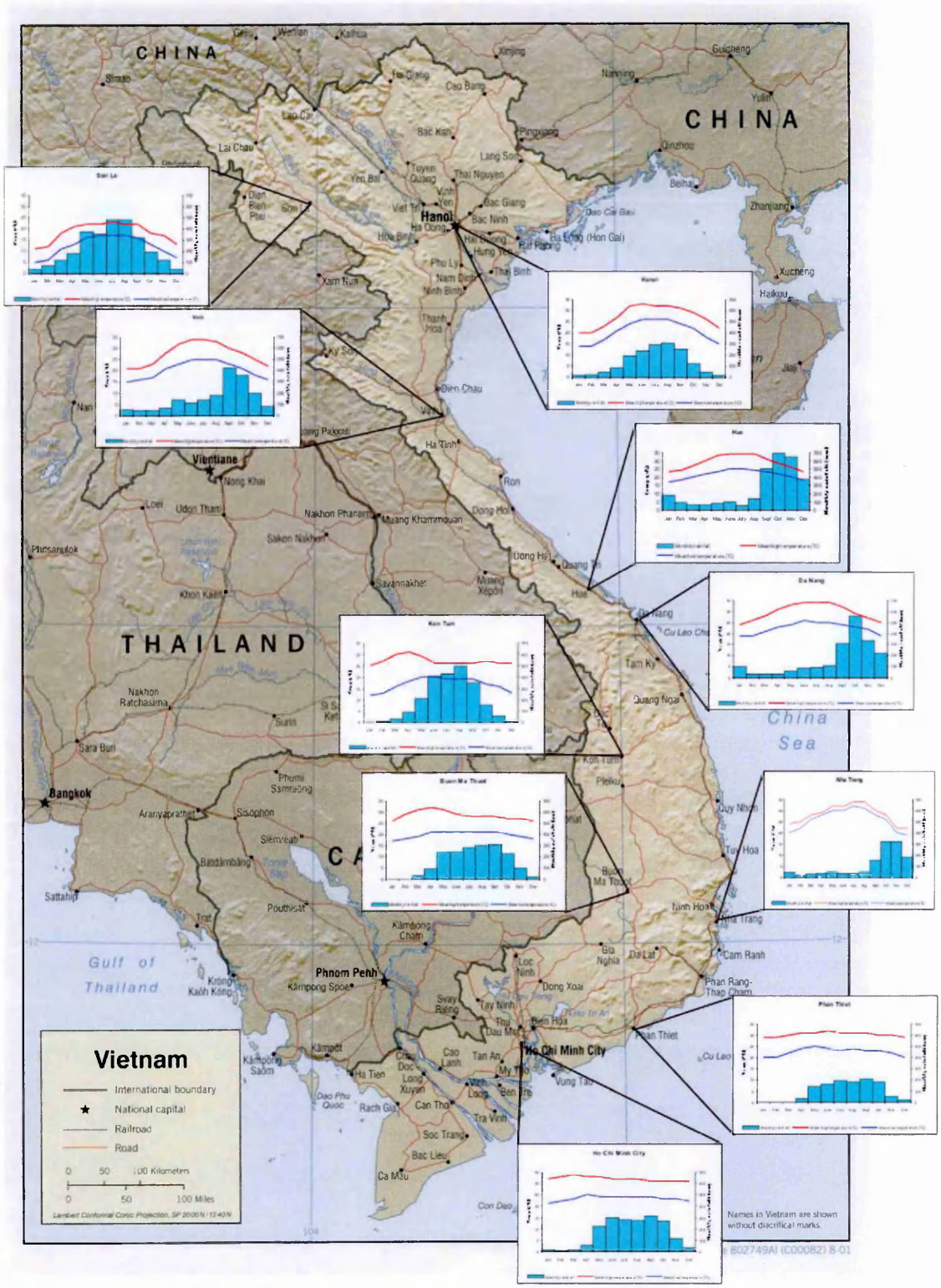
Ethnic group	Total population
Kinh	65,795,718
S'tiếng	66,788
Tày	1,477,514
Nùng	856,412
M'Nông	92,451
Dao	620,538
Hoa	862,371
Ê Đê	270,348
Rac Lay	96,931

Table 2.1 National populations of the ethnic groups included in study projects (1999 Census)

The Ê Đê (also known in the anthropological literature as Rhade) are the most numerous of the Malayo-Polynesian speaking ethnic groups, and became a force in a "highland elite" during the 19th and 20th centuries. There was some intermarriage with other ethnic groups during this period, but only at the top level of society. The majority live in the current day provinces of Đắc Lắc and Gia Lai. The Rac Lay (sometimes written Roglai), also Malayo-Polynesian speakers, are a small and traditionally peaceful group. There is a suggestion that they too were once organised along clan lines, but these had degenerated to the extent that they are barely touched upon in the oral history recorded by French anthropologists in the 19th century. There is also some evidence that the closely related Chru ethnic group was formed by intermarriage between remnants of the disintegrating Chăm empire and the Rac Lay, suggesting that they have historically adhered to reproductive taboos less rigidly than some other minority groups.

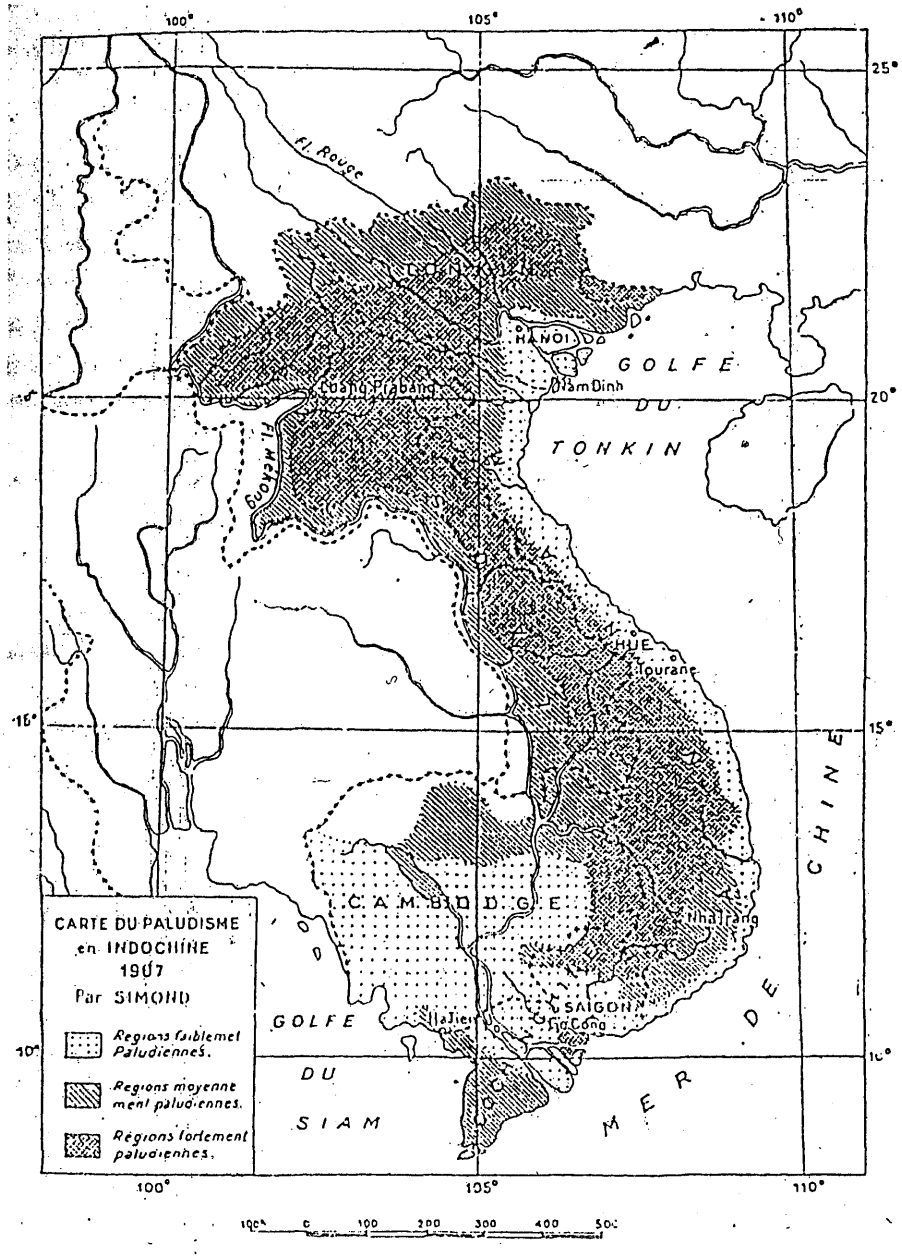
Administrative and healthcare structure

The healthcare structure in Vietnam generally follows the administrative structure, particularly outside the major urban centres. The highest administrative division in the country is the province, and each province will have a provincial health services department, usually one major provincial general hospital, a provincial malaria control programme, and a provincial public health department. All these branches of the health service are integrated at the district level (the second administrative tier), so the district hospital will also be the district health services office (and the directorial roles will be combined), and within the hospital will be the local malaria control and public health team, and the local women and children's health team. Each commune (the next level down) will have a health station. These will usually be staffed by a doctor, midwife or clinical assistant, or a combination of two of these individuals from different backgrounds. Whatever their level of training, they will have to fulfil all roles in the health station. Most health stations have between 2 and 6 beds for short term inpatient treatment, and will be supplied with essential drugs. Patients must pay for all consultations and treatments with a few (sometimes theoretical) exceptions, such as antimalarials and treatment for ethnic minority patients. The village health station is also responsible for overseeing the work of the village health workers of whom there is one in each hamlet (the smallest administrative unit). There is also a plethora of private healthcare outlets, from local general stores, which sell cocktails of drugs in small packets, to any health care worker (excluding the village health care workers) setting themselves up to provide a private service. As these will usually be the same people as staff the government services, there can be a conflict of priorities.

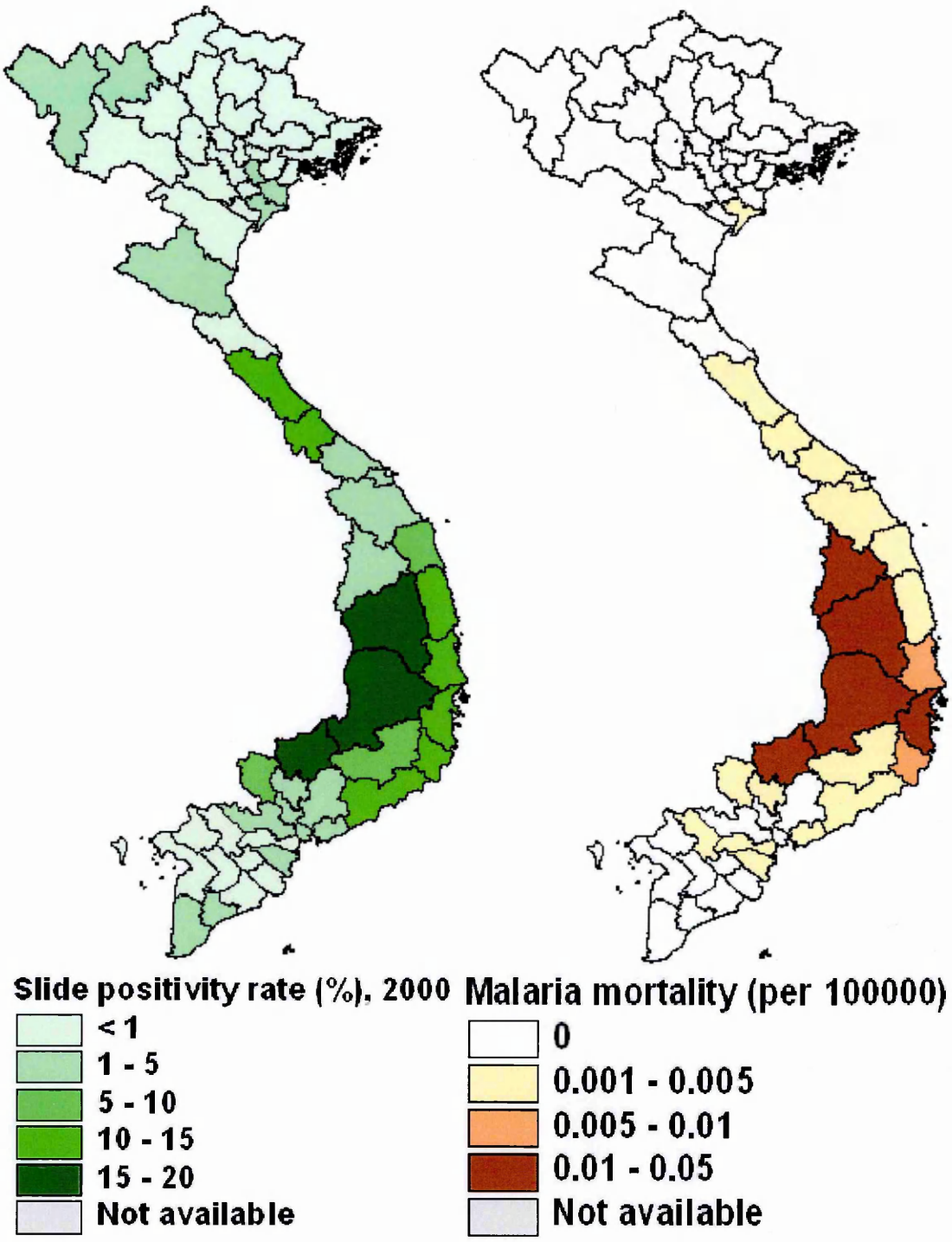


Map 2. Map of Vietnam in relief with climate data for selected significant points. Climate data is given per month, and includes maximum temperature (red line), minimum temperature (blue line), and monthly rainfall (shaded blue bar). All graphs use the same scales (0-35ºC in increments of 5ºC on left axis, 0-700mm rainfall in increments of 100mm on right axis)

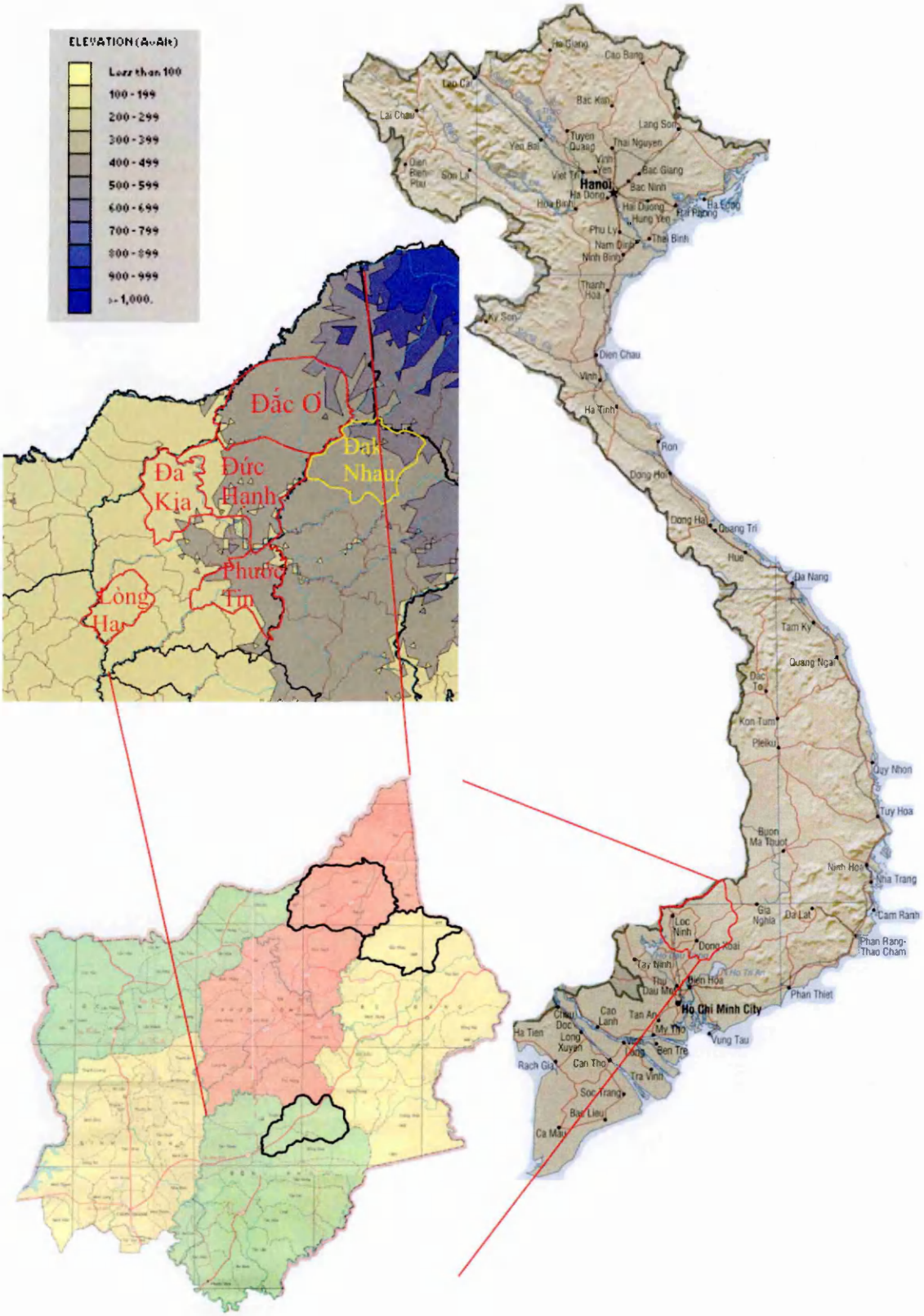
Fig 2.1. Pictures of typical study site terrain and vegetation



Map 3. Early 20th century map of malaria in Indochina



Maps 4. Malaria mortality and slide positivity rate in Vietnam in 2000



Map 5. Map of the study area: Phước Long district in Bình Phước province, southern Vietnam. Study communes for the first survey are outlined in black in the province map, and communes involved in other studies are outlined in red in the district elevation map (Đak Nhau, outlined in yellow, was one of the first survey communes only).

Malaria

Entomology

The major vectors in the study region are *Anopheles minimus* and *Anopheles dirus*. *An.minimus* is an endophilic resting species, which bites indoors and out and has no preference for human or animal victims. *An.minimus* requires clean, slow flowing small to medium sized streams or small, clean, regularly refreshed pools for breeding. *An.dirus* is an exophilic resting, forest dwelling species with a preference for biting humans both indoors and out. *An.dirus* requires shady pools regularly refreshed by rainwater to breed in, and as such is predominantly a rainy season vector. Entomological surveys in other, similar regions of Vietnam have shown a much higher prevalence of sporozoites among *An.dirus* captured in deep forest, where transmission is presumably maintained by small groups of unprotected forest workers. *An.dirus* is said to be a late biter, mostly between midnight and 3am, although has been recorded to bite much earlier. *An.minimus* is said to be mostly crepuscular, with biting peaks from 6pm to 9pm and 3 to 6am. The importance of biting times documented at sites distant in space or time from an area under study is unclear, as there is some evidence that mosquitoes alter their feeding habits according to local circumstances. Other species, such as *An.sinensis*, *An.sundiacus* and *An.maculatus* have previously been implicated as either minor vectors or vectors in specific regions which are no longer malarious.

Geographical trends

Malaria in Vietnam, and "Indochina" in general, has always been associated with the highlands (see terrain below). The early 20th century map (map 3) of malaria risk in Vietnam demonstrates this clearly. This is likely to be due to the preference of two of the main vectors discussed above. The situation appears similar in 2000 (map 4): currently the most malarious provinces are Bình Phước and Đắc Lắc in south central Vietnam (data from the National Institute of Malariology, Parasitology and

Entomology, Hanoi (NIMPE)), provinces characterised by low highlands, significant numbers of ethnic minority peoples, and receivers of large numbers of economic migrants in search of land from non-malarious areas in the north of Vietnam.

Temporal trends

There has been a rapid decline in all malaria indicators over the past 20 years. In 1991, there were over 4,500 deaths from malaria in Vietnam. In 2000, there were 150 (NIMPE). Malaria prevalence has shown a similar fall, to the extent that once malarious areas, particularly in the northern highlands and coastal regions, are now virtually free of malaria. This fall is due to a combination of four factors. The introduction of artemisinin derivatives has probably had the most profound effect on mortality, and their local production, widespread availability, and adoption into national guidelines as the first line treatment for *P. falciparum* malaria is likely to have contributed significantly to the decrease in transmission. The high priority placed on malaria control by central government appears to have convinced many families with sufficient funds to purchase bed nets, and a number of projects, mostly externally funded, have distributed thousands of bednets to poorer households. Most of these nets are treated, and central government funding has sustained an ongoing retreatment programme since the early 1990's. Good data on the combined impact of adequate diagnosis and treatment and the distribution of insecticide treated nets in the Vietnamese setting comes from an exquisitely well run NGO project in the central highlands. Medische Comité Nederland-Vietnam (MCNV), in collaboration with NIMPE, ran an insecticide treated net (ITN) implementation programme, with detailed epidemiological run in and monitoring, in Khánh Phú commune, Khánh Vĩnh district of Khánh Hòa province, near the central coastal city of Nha Trang, between the years of 1994 and 2000, and have continued malaria surveillance since then. The smear prevalence has dropped from 35% to 10%, and calculated EIR from 35 to 2. *An.minimus* has virtually disappeared and *An.dirus* numbers and sporozoite rate

diminished markedly. Sporozoite positive *An.dirus* are now usually only caught on collection trips to the deep forest. The dramatic deforestation of the last 20 years is also likely to have made a significant contribution to malaria's decline. Vietnam has lost approximately 30% of its primary forest cover during this period (and over 70% of richly stocked forest) (Vietnamese Forest Inventory and Planning Institute, cited in the United Nations Environment Programme (UNEP) State of the Environment in Vietnam Report, 2001). Most of this clearance is for sedentary farming or firewood collection rather than commercial logging, particularly in the study region, and several areas along the Vietnamese-Cambodian and Vietnamese-Laotian borders are designated as "most under threat from agricultural pressure" by UNEP. The fourth and final factor is the dramatic improvement of socioeconomic status over this time. The changes in behaviour, small town "urbanisation", and access to health care that have accompanied this development are likely to have contributed to the continued fall in transmission, although are difficult to quantify with any degree of precision, particularly in retrospect.

Malaria and ethnicity

The geospatial correlates of malaria in Southeast Asia at a macroepidemiological level were discussed above. Given the predominantly upland distribution of ethnic minority groups in Vietnam, it is inevitable that they will have a larger burden of malaria than the majority Kinh in nationally aggregated data. Local malariologists report that this discrepancy persists amongst individuals of different ethnic groups living in close proximity in highland areas. Published documentation of this phenomenon exists for Thailand (Tiensuwan 2000), and the central highlands of Vietnam (Luxemberger – unpublished data). No such comparative data exists for southern Vietnam, although separate surveys in majority and minority groups in the 1960's, together with a contemporary seroepidemiological study, suggest this might have been the case at that time (Bowman et al. 1971; Colwell et al. 1971).

Haemoglobinopathies in Vietnam

Haemoglobinopathy prevalence

Whilst a number of publications have documented the prevalence of haemoglobinopathies in Vietnamese subjects, good quality, community based data is sparse. Three individuals (2.7%) in a survey of 114 inpatients of a Saigon hospital had HbE (Albahary et al. 1958), but the patients were admitted "for haematological problems or other illnesses", suggesting a biased population. A subsequent survey of 482 inpatients at Chợ Quán hospital (which, coincidentally, became HTD) found a similar prevalence of 3.53% (Blackwell et al. 1965), and as these subjects had all been admitted for diarrhoea during a cholera outbreak, the population is less likely to be biased. Neither report specified the ethnicity of the subjects: very few are likely to be highlanders, but a significant proportion might be Chinese rather than Kinh. A number of studies of Vietnamese refugees similarly do not report ethnicity: 1/79 Vietnamese subjects screened on first attendance at a northern California hospital carried HbE, and 6 (8%) carried β-thalassaemia (Hurst et al. 1983). The subjects were from 36 families and no comment was made on the number of families with β-thalassaemia. A rather more "population based" refugee survey from the US found 2% of 116 Vietnamese carried HbE and 3% β-thalassaemia (Monzon et al. 1986). Once again no details are given on the family relationships of the individuals with haemoglobinopathies, and the 778 subjects in the survey were drawn from only 182 families. Although the Vietnamese in this report were not definitively reported as Kinh, 206 Tai Dam subjects (of whom HbE was detected in 11% and β-thalassaemia in 8%) are reported separately, suggesting the Vietnamese are Kinh. The high proportion of Tai subjects with HbE again raises questions about the ethnic classification system and family structures of this population, although the authors stress that "records were maintained to determine the family relationship so that the final results were not biased by inclusion of closely related persons". Another possibility is that these Tai were Laotian rather than

Vietnamese (although Vietnamese Black Tày outnumber Laotian Black Tày by 14:1- http://www.ethnologue.com/show_language.asp?code=blt), in which case assimilation might have introduced HbE into the Tày. The authors of a report on haemoglobinopathies amongst 810 Vietnamese refugees in Thailand excluded "Vietnamese with Chinese or other ethnic origins", implying the subjects were all Kinh. This survey found 17 HbE heterozygotes (2.1%) and 20 β-thalassaemia heterozygotes (2.5%) (Pornpatkul et al. 1980). Inclusion of ethnic minorities is likely to be responsible for the elevated estimates of HbE (8.9% in southern Vietnam, and a more likely 3% in the north and centre) reported by De Traverse et al at the 7th Congress of the European Society of Haematology, cited in both (Flatz 1967) and (Fucharoen et al. 1987a), but unavailable for direct examination. The 225 subjects in the south are listed as Kinh in the Fuchareon review, however. The prevalences of β-thalassaemia of 1.5% and 1.7% must therefore also be regarded with suspicion, despite their feasibility. Only 4 out of 127 individuals requiring a visit from a community medical team in central Vietnam carried HbE (Anderson 1966), although once again ethnicity was not specified. A study of medical students and blood donors in Hồ Chí Minh City (HCMC) showed a prevalence of HbE of 1.3% in 153 subjects said to be from north Vietnam, 0 in 35 from the centre, and 3.2% in 221 from the south (Le Xuan et al. 1968). Ethnicity was not specified. Six out of 17 "Montagnards" (a generic term for highland dwelling ethnic minorities) had HbE in a survey of south Vietnamese military volunteers, including two homozygotes, but precise ethnic affiliations are not offered (Blackwell et al. 1972). The same survey found a prevalence of 4.2% among 307 Vietnamese volunteers, including 1 homozygote.

A number of the Vietnamese language studies which have examined the distribution of haemoglobinopathies are not true prevalence studies, reporting the experiences from one centre, or results from a diagnostic laboratory (Vu 1999). Others have severe methodological weaknesses, such as a prevalence survey of beta globin anomalies

among 147 S'tiếng subjects, which found 61 HbE heterozygotes and 9 HbE homozygotes, but also reported 10 β-thalassaemia-HbE compound heterozygotes despite finding no β-thalassaemia heterozygotes (Le et al. 2002). The glaring anomaly in this result was not discussed by the authors, who made specific mention of the fact they did not record the ages of their subjects. Given the use of levels of HbF to diagnose the compound heterozygote, this is a severe omission, but not thought worthy of comment. In addition, no mention was made of family structure within the sample, although sadly this failing is repeated in almost all prevalence studies, including all smaller studies where the effects are likely to be greater. The Fuchareon and Winichigoon review quotes two Vietnamese reports as primary sources for HbE and β-thalassaemia prevalences in Vietnam, neither of which were available for critical appraisal (Bach Quoc Tuyen et al. and Nguyen Cong Khanh et al. in Fucharoen et al. 1987a). Their data is reprised in table 2.2 with a rider that methodological weaknesses might render these figures meaningless. The Ca Tu (Cơ Tu, Katu) and Pako (Pacoh, Paco, Pokoh) and Vân Kiều (sometimes Bru), are linguistically closely related Mon-Khmer speaking groups.

Ethnic group	Haemoglobin E		β-thalassaemia	
	Number	Carrier frequency	Number	Carrier frequency
Kinh (Hanoi)	401	1.2%	401	1.5%
Kinh (North)	512	0.95%	512	1.2%
Minority ethnic groups (unspecified)	346	2.3%	346	12.4%
Tày	199	1.0%	199	11.0%
Mường			40	25.0%
Ê Đê	371	41.7%	371	1.3%
Pako	228	6.1%	228	8.3%
Vân Kiều	78	23.1%	78	2.6%
Ca Tu			?	14.0%

Table 2.2. Prevalences of HbE and β-thalassaemia reported in studies from Vietnam cited in secondary sources

One surprise result in this table is the relatively high prevalence of both HbE and β-thalassaemia in the Pacoh. It would be tempting to put this down to the above mentioned methodological issues, were it not for a reasonable study amongst the Mường of Hòa Bình province, which documented a carrier frequency of 12% for HbE and 20.6% for β-thalassaemia (Bui 1999). The prevalences of β-thalassaemia trait in the Mường reported in these two studies are the highest in the world. The Bui study found 1.9% of 605 Kinh had HbE and 5.3% β-thalassaemia, suggesting that the estimate of β-thalassaemia might be a little high (possibly due to a poorly chosen and overly aggressive cut-off value for the HbA2 percentage indicating β-thalassaemia trait), but even if only 10% of Mường carry β-thalassaemia, this would still be an extremely high prevalence for an ethnic group with 10% HbE. There are no particular characteristics of the Mường which might explain this finding: they are a sizeable ethnic group, estimated population 1,137,515 (1999 Census, Vietnam Office of National Statistics); they are linguistically related to the Kinh in some schemata (http://www.ethnologue.com/show_family.asp?subid=90160), and their traditional home is in the highlands of north and north central Vietnam.

The best data for HbE in southern Vietnamese ethnic minority groups comes from a 1965 study by Bowman and colleagues, which demonstrated gene frequencies for HbE of 0.025 amongst 247 Kinh, 0.209 in 139 Khmer, 0.212 in 65 Ê Đê, and 0.365 in 49 S'tiếng (Bowman et al. 1971). Whilst numbers are small, the exclusion of first degree relatives in this study results in more precise estimates of prevalence than many other surveys of similar size. A point of interest is the low frequency found in the Sedang (0.029), a Mon-Khmer speaking group linguistically closely related to the S'tiếng, but geographically separated by hundreds of kilometres and the interposed Malayo-Polynesian speakers.

Several of the refugee studies mentioned above also offer estimates of the prevalence of α-thalassaemia. Without exception this is calculated from red cell indices and haemoglobinopathy data, however, which will grossly underestimate the prevalence of single gene deletion mutations, but may overestimate total prevalence unless studies of iron status are also included. One survey of cord blood HbBarts found a prevalence of 2.5% amongst 201 newborns in Hanoi (Nguyen et al. 1992). No ethnic details were given, although the mothers are likely to be overwhelmingly Kinh.

Project Design and Conduct

The aim of this programme of research was to tackle all the clinical and epidemiological aspects of the relationship between HbE and malaria. This entailed surveys of malaria prevalence and haemoglobinopathy prevalence in our study area, a case control study of severe malaria and HbE, and a birth cohort assessment of the incidence of malaria infections. The remainder of this chapter will present the study designs together with theoretical considerations involved in their planning, followed by a description of the project infrastructure and certain important problems encountered during the execution of the studies, and finally the laboratory methods used.

Choice of study venue

HbE is highly prevalent only in Southeast Asia and Assam. Vietnam was selected as the most suitable host country in these regions on the basis of continuing malaria transmission, an established collaborative programme of infectious diseases research, capable of initiating and sustaining complex community based studies, and a preliminary haemoglobinopathy prevalence survey which identified a number of ethnic groups with high gene frequencies of haemoglobin E.

Bình Phước was selected as the site of the clinical studies by virtue of being the closest of Vietnam's three most malarious provinces to HCMC. Within Bình Phước, Phước

Long was selected following the first survey as the district with the highest prevalence of both malaria and haemoglobin E.

Project infrastructure:

The Hospital for Tropical Diseases and Oxford University/Wellcome Trust Clinical Research Unit, Hồ Chí Minh City

The collaboration between the Hospital for Tropical Diseases (formerly the Centre for Tropical Diseases), Hồ Chí Minh City, and Oxford University was established in 1990. Funded by the Wellcome Trust, its initial remit was to examine the effectiveness of artemisinin derivatives in severe malaria. The size and focus of the unit have grown considerably: current major areas of interest are bacterial meningitis, typhoid, dengue, tetanus, Japanese B encephalitis and TB. The strength and success of this collaboration, and the skill and motivations of the key players, were essential in negotiating the studies through the minefield of Vietnamese political and healthcare priorities. Despite major involvement in projects in 5 provinces of southern Vietnam, all unit facilities and staff are based in HCMC.

The Hospital for Tropical Diseases acts as a primary and secondary centre for nearby population, and a tertiary referral centre for infectious diseases in southern Vietnam. In addition to the 4 adult and 4 paediatric wards there is an acute malaria ward, an adult HIV ward, a paediatric ICU/HDU, a general adult ICU/HDU, and dedicated ICU's for tetanus and malaria. Those clinical elements of the studies carried out at HTD recruited patients on the PICU and malaria ICU.

Phước Long District Health System

Phước Long district health centre (hospital) is located in Thác Mơ town. There are 70 inpatient beds, and admits 850 patients per year from 10,000 outpatients. It is equipped with a microscope, an X ray machine, an ultrasound machine, a manual biochemistry analyser and a centrifugal (QBC) blood count machine which was rarely

used due to the cost of the disposables. During the study period, a coulter counter was donated to the hospital by the European Commission (EC) malaria programme, but was rarely used due to cost considerations, and most haematological assessments were performed manually. The X ray machine was also replaced by an aid agency one year into the study. The study donated a new microscope, and also supplied an incubator, biochemistry reagents and other lab consumables for the duration of the study. The hospital is equipped to perform emergency and straightforward elective surgery, administer oxygen and intravenous therapies, and possesses one extremely old ventilator which has been used on occasion for patients ventilated in other hospitals whose relatives want to transfer them closer to home. Despite this, patients are rarely intubated for resuscitation or transfer. There are no blood transfusion services, and these and other services that Phước Long cannot deliver are provided at the district hospital, approximately 1 hour away by road. The hospital is staffed by 10 doctors, 4 midwives, 4 clinical assistants and 40 nurses. The local malaria control and public health team consists of 6 people, with a clinical assistant as leader, and the local women and children's health team consists of 7 midwives.

The health stations in the 5 communes in which most of our community studies are based are staffed by a doctor and a midwife (Đa Kia, Đức Hạnh and Lòng Ha), or a clinical assistant and a midwife (Đắc Ơ and Phước Tin).

Every hamlet has a community health volunteer, and there is one western trained community midwife in Đức Hạnh commune, and one traditional midwife in Phước Tin.

General conduct of the studies

All studies were carried out in collaboration with Vietnamese partners. With the exception of Dr Triết and Dr Thái, recruited to the programme in 2001, and Miss Tâm and Mr Minh, our data entry clerks, it was not permissible for the study to employ anyone directly. We had to rely instead on the local health care services to recruit

additional study staff as necessary, and reimburse local health care workers for time spent on the study via their existing management structure. All line management was similarly devolved. The main collaborators were the Hospital for Tropical Diseases, Hồ Chí Minh City (HTD), the Institute for Malariology, Parasitology and Entomology, Hồ Chí Minh City (IMPE), the Health Service of Phước Long District and Bình Phước Provincial Hospital. Other key parties were the Bình Phước Provincial Malaria Control Programme and the Health Service of Bình Phước.

Ethical approval

All studies were approved by the Ethics Committee of the Hospital for Tropical Diseases, Hồ Chí Minh City, although as the cross sectional surveys differed little in design, ethical approval was not sought for each survey separately. The studies were designed as the Oxford tropical research ethics committee (OXTREC) was being established, and prior to the requirement for all studies to have dual ethical approval. The HTD ethics committee was keen to establish that the study was of scientific interest and properly designed, and, in particular, that any potential risk or harm to participants was minimised. Changes to the protocols insisted upon by the committee for these studies focussed on reducing the frequency and volume blood taking from children. The implementation of informed consent was hotly debated, however: Vietnamese society does not value individual autonomy highly, and all interactions with authority figures involve being told what to do, rather than being asked what one wants to do. The practicality of obtaining informed consent for genetic studies in an uneducated population was also disputed. Discussions with community leaders did not unearth the sort of suspicions and fears of genetic studies which have been raised in certain parts of Africa. The cross sectional surveys and KAP study were conducted under the auspices of IMPE-HCMC. There was considerable opposition to any sort of formal consent process for these studies, on the grounds that such surveys were the normal business of the malariology institute, which they had a right to conduct in these communities, and

that obtaining written consent would set an undesirable precedent. Concerns were also raised that seeking written consent in the prevailing political and social climate in Vietnam would affect the accuracy of responses in the KAP study, as making the whole process appear more official would lead to a variety of perceived incentives to misreport certain information, particularly concerning income. Details of consent processes for individual studies are given in their respective sections of this chapter.

Survey design

All the surveys were conducted by staff from HTD in collaboration with IMPE, HCMC. The design of the surveys evolved as increasing experience of the practicalities of working with all interested parties was gained, and particular issues with the data obtained became apparent. There was also some variation in the aims of the surveys, with consequent differences in data and samples collected. Individual consent was not sought from each study subject in the surveys (see above), which had nevertheless been discussed with community leaders in advance. Implied consent was inferred from individuals attending the survey in response to the invitations issued.

Initial survey

Aims

Bình Phước province had been selected for the study as the only region within a manageable distance of Hồ Chí Minh City with persistent malaria transmission and a relatively high proportion of ethnic minority groups likely to have a high prevalence of HbE. Existing malariometric data was limited to slide positivity rates from health stations and the occasional, mostly small, community survey. The limited data on haemoglobinopathies has been outlined above. Two features of malaria epidemiology in the region were portrayed as axiomatic by local doctors, but no data existed to formally corroborate these opinions: namely that malaria transmission declined with distance from the Cambodian border, and minority ethnic groups suffered a much

higher burden of malaria. In addition to gathering more extensive malaria prevalence data with finer granularity in respect of geospatial and demographic variables, and expanding the haemoglobinopathy prevalence dataset both in size and scope (to include the common alpha thalassaemia mutations), the initial survey provided an opportunity to develop an understanding of factors crucial for future study design. The phenomenon of asymptomatic parasitaemia and its importance in formulating a case definition of clinical malaria has been mentioned in Chapter 1, thus a third aim of the survey was to obtain as much information as possible on the symptoms and signs of febrile children and adults with and without parasitaemia. An appreciation of the patterns of health care seeking behaviour of the local population, for both peripartum care and in case of febrile illness, was felt to be important in planning the cohort study in particular, so questions aimed at elucidating these patterns were included.

Study sites

Three communes in different districts of Bình Phước were selected for the survey: Đồng Tâm commune in Đồng Phú district, Đak Nhau commune in Bù Đăng district, and Đắc Ơ commune in the above mentioned Phước Long district (see Map 5). Đồng Phú is adjacent to the provincial capital, Đồng Xoài, Đồng Tâm commune lying approximately 20km distant from the town. Previously almost exclusively populated by S'tiếng, migration of Kinh since 1975 has displaced the S'tiếng to the more remote areas, and they now comprise just 25% of the commune population. Đồng Phú lies at the base of the first foothills of the Trương Sơn mountain range, at altitudes of less than 200m. In common with Phước Long and Bù Đăng, most of the land is under cultivation with rubber, coffee, cashew, pepper and cassava, with pockets of paddy. Unlike the other two districts, there is little remaining primary or secondary forest. The commune health station is staffed by a midwife and a doctor, and there are facilities for microscopic diagnosis. The district health station lies some way from the commune, however, and the secondary referral path would be to the provincial hospital at Đồng Xoài. Đak Nhau

and Đắc Ơ communes lie adjacent to one another across the district border. Both are significantly more remote than Đồng Tâm, with roads that often become impassable to 4 wheeled vehicles during the rainy season. Both have health stations staffed by a clinical assistant and a midwife, and also have facilities for the microscopic diagnosis of malaria. Đak Nhau lies some 20km distant from the district health centre, whilst Đắc Ơ is 40km from Thác Mơ town, the district capital of Phước Long. Rolling foothills are occasionally sufficiently steep to impede easy access, though there is little uncultivable land. There are a number of permanent streams, which can become impassable in the rainy season, and Đak Nhau lies at the eastern border of an artificial lake created by the hydroelectric dam at Thác Mơ town. Đắc Ơ has a border with Cambodia, whereas Đak Nhau does not. Đak Nhau marks the western extent of the M'Nông people, who comprise the largest minority group in Bù Đăng, whereas Đắc Ơ is approximately 40% S'tiếng, with 10% Tày Nùng and 50% Kinh. Similarities and differences between the M'Nông and the S'tiếng have been outlined in the introduction. The survey covered all hamlets of these 3 communes, and was carried out sequentially in Đồng Tam, Đak Nhau and Đắc Ơ between February 15th and March 7th 2000, lasting a week in each commune.

Survey subjects and design

A large sample was necessary in order to achieve all the aims of this survey, in particular the elucidation of the relationship between parasitaemia and symptoms. In view of the variation of this association with age, particularly through childhood, we enriched the sample with children. The sample size requested of the survey teams was 50 individuals in each of the following age groups from each of the 3 study communes: every year until the age of 10, every 5 years from the ages of 10 to 20, every 10 years from the ages of 21 to 70 (a total of 2550 subjects). Initial discussions had focused on performing a cluster survey. Previous practical and political difficulties encountered during the 1999 cluster survey, considered with the large sample size, led to a different approach being followed. The commune authorities and health station were asked to

arrange the allotted number of attendees. This number was divided between the hamlets, and the hamlet community health workers/volunteers (y tế thôn bản – YTTB) requested to invite that number of individuals from the different ethnic and age groups. An attempt was made in Dak Nhau to invite specific people from the commune population lists. A more general invitation was issued in Đắc Ơ and Đồng Tâm.

Survey conduct

The survey was carried out by staff from IMPE-HCMC and HTD. The survey form is presented in Appendix 1. Three teams operated at separate sites in the commune, changing venue at lunchtime. I was permitted to travel to the survey sites to supervise on a daily basis. Despite leaving the city at 5am, the 4 hour journey resulted in limited time with the teams in the field. Each subject was administered the questionnaire, examined, with close attention paid to the presence of anaemia, jaundice or splenomegaly, and had his or her temperature measured. Pyrexial subjects were questioned in more detail about any symptoms which might explain their fever, and examined more closely for the presence of an upper respiratory tract infection (URTI) and other likely causes of fever (although it soon became apparent that otoscopy was rarely performed). Finger-prick capillary puncture was performed (heel prick in young infants) using cutting lancets designed to deliver a high blood volume (FreeFlow, Beckton Dickinson), a thick and thin blood film prepared, and 250-1000µl blood gathered into EDTA containing tubes using a proprietary collection system (Greiner UK). Any individual clinically suspected of having malaria was tested using an HRP2 antigen kit (either Parasight F or Paracheck), and the decision to treat based on a combination of clinical assessment and this test result. The teams carried supplies of analgesics, antipyretics, vitamins, antihelminthics and a limited range of antibiotics to provide treatment for any coincidental ailments discovered on examination. Study forms, blood samples and slides were labelled using pre-prepared, sequentially numbered adhesive labels.

Sample handling

Samples were kept on ice in the field, and transferred to polystyrene containers filled with dry ice once or twice per day, depending on the remoteness of the morning location. Samples were stored on dry ice for 1-3 days until transfer to HTD, where they were stored at -20°C pending shipment to Oxford for haemoglobin typing. These samples were shipped whole in March 2000. The methods employed for haemoglobin typing by HPLC and PCR characterisation of alpha globin anomalies are described below and detailed in Appendix 3. Thin smears were fixed in the field prior to transportation to HTD, where thick and thin smears were read as described below.

Subsequent surveys – general considerations

By the time the second survey took place in August 2000, Phước Long district had been selected as the site of all future studies. All subsequent surveys were conducted in three communes of Phước Long, one of which, Đắc Ơ, had been included in the first survey. Important lessons learned from the initial survey were the need for a list of household members, in order to be able to delineate family relationships, the difficulties inherent in using a study form which included a decision branch point, and the futility of insisting on the acquisition of certain pieces of data when they were not being collected after the second reminder. These experiences prompted a census prior to the second survey, and a complete restructuring of the survey form, which then remained similar for subsequent surveys. The conduct of the surveys was essentially similar throughout, with minor variations in type and number of samples taken, and the follow up exercise incorporated into the second survey. Variations in aims, data and samples collected, sample handling and analysis and sampling strategy are dealt with by survey.

Managing data from the surveys, in particular ensuring that subjects were linked to the census data, proved a significant challenge. The data management methods are

described in the overall data handling section below, as some prior understanding of the issues discussed in the "specific difficulties" section is essential.

The census

A census was effected by requesting a list of family members of all households from the hamlet community health volunteers in all 41 hamlets in the 3 communes. No list was ever received from one of the hamlets in Đắc Ơ (Đak Lim). The lists from the other hamlets varied in quality, even on first inspection, with some including little age and sex data, and it became apparent during the ensuing years that many families had been omitted, and others were incomplete. Nevertheless these lists provided a reasonable basis for associating subjects into families, and, subsequently, an almost adequate sampling frame on which to base the survey samples.

Second survey

Aims

The disappointing quality of data gathered on symptoms and parasitaemia suggested the need for a follow up exercise 2-3 weeks after the survey to ascertain how many of the parasitaemic individuals had developed symptoms. The lack of a properly constructed sampling frame or adequately defined sampling method may have biased the initial estimates of smear positive prevalence. This survey aimed to obtain a more valid estimate.

Survey sites and subjects

This survey was carried out in all 11 hamlets of Đắc Ơ, 14 of the 18 hamlets in Đức Hạnh, and all 12 hamlets of Đa Kia. The sample size was set at 800 individuals per commune, divided equally between the hamlets, with no enrichment of any age or ethnic group. This limit was set pragmatically rather than scientifically, being the largest number the teams felt they could cope with. The aim was to obtain sufficient numbers of smear positive individuals to be able to obtain reasonable estimates of the

proportions of symptomatic, presymptomatic and asymptomatic smear positive individuals, and make meaningful comparisons between them.

Survey conduct

The initial survey was conducted between the 10th and 31st of August 2000 in a similar fashion to the first survey, with a revised data form (appendix 1). The survey design had envisaged follow up of all smear positive subjects approximately 2 to 3 weeks after the survey, but the actually delay was longer. Subjects were followed up at home, and questioned on the presence of fever, rigors, chills or headache in the period between the survey and follow up visit. Axillary temperature was also measured and the spleen palpated.

Sample handling

Experimentation with various storage conditions on volunteer blood at HTD had revealed no differences in quality of HPLC result between keeping samples frozen, refrigerated, in ambient shade or even sunlight for periods of up to 24 hours. The samples from this survey were kept on wet ice in the field, then at 4°C for an extended period pending DNA extraction. When this became impossible for temporary logistical reasons, the samples were transferred to a -20°C freezer. An aliquot of haemolysate was sent to the UK for HPLC typing in July 2002.

Third survey

Aims

The documentation of the prevalence of glucose-6-phosphate dehydrogenase (G6PD) deficiency in the study population was necessary to anticipate the potential magnitude of its confounding effect. Formal assays of G6PD activity were felt to be unfeasible. The kind offer of rapid test reagents from Professor Hiroyuki Matsuoka of Jichi Medical School, Japan allowed the qualitative assessment of G6PD status in the field. Despite an improved sampling strategy in the second survey, the age and sex distribution still

deviated from that expected in the population (see Chapter 5). Researchers attached to the project in Oxford were hoping to extend the HbE haplotype, for which families were necessary. The invitation of entire family units was hoped to achieve both these aims. This survey was conducted at the end of the rainy season, and would establish a baseline for estimating the magnitude of any seasonality in prevalence.

Survey subjects and sites

The survey was conducted in the same hamlets as the second survey. A similar sample size was aimed for, although the clustering of subjects in households would result in less precise estimates of malaria prevalence. Households were selected locally in the fortnight prior to the survey, and local health workers invited all household members to attend the survey. 800 unrelated adult individuals were selected during the survey to have their G6PD activity assayed. These individuals were to be Kinh males or S'tiếng individuals of either sex.

Survey conduct

The survey was carried out between the 12th and 28th December 2000. The study form was modified to elicit a history of haemoglobinuria or tetanus (the latter in order to be able to use some subjects as controls for other projects (appendix 1)). The study conduct was essentially similar to previous surveys. EDTA samples selected for G6PD analysis were transported to the commune health station within 4 hours of being taken.

Sample handling

Samples for G6PD assay were kept at room temperature until this test had been completed, when they were stored at 4°C until transport to HTD. All other samples were kept on wet ice until transfer to HTD. Samples from G6PD deficient individuals were aliquoted, and haemolysate only sent to Oxford for HPLC analysis. All other samples were stored at 4°C until transport to Oxford in their entirety.

Fourth survey

Aims

The family sampling strategy of the third survey was utilised in this survey in order to provide consistency in estimates of malaria prevalence across surveys over this calendar year and hence assess the existence of seasonality in transmission. Interest in the potential interaction between HbE, anaemia and parasitaemia led to the inclusion of haematocrit measurement.

Survey subjects and sites

The survey was conducted in the same hamlets as the third survey. The desired sample size was reduced to 500 individuals per commune on the basis of concerns that the excessive workload was leading to many of the data collection difficulties persistent throughout previous surveys. Families were identified and invited as in the third survey.

Survey conduct

The survey was carried out between 27th March and 3rd April 2001. A minor change to the survey form was the elimination of "Traditional physical therapy" as an option for previous treatment (appendix 1). The survey was conducted as previous surveys, but in addition a 75µl haematocrit tube was filled during capillary blood sampling. Due to the social unrest in Đak Lak province, no foreign member of the study team was permitted to visit the field during this survey.

Sample handling

The haematocrit tubes were kept at room temperature. Samples collected on all but the last day of the survey were spun and read in the field on the evening of collection, whilst those taken on the last day were transported to HTD and spun and read the following morning. The EDTA samples were kept on wet ice in the field, and at 4°C once at HTD. After several weeks they were spun and separated. An aliquot of red

cells was prepared for HPLC analysis, then plasma, cells and haemolysate were frozen at -20°C. The haemolysates were sent to Oxford in June 2001.

Fifth survey

Aims

The estimates of G6PD deficiency amongst the S'tiếng was considerably higher than that verbally reported from a small study using the Sigma fluorescent spot rapid test in the south of Phước Long district. Confirmation of our findings by a formal quantitative method was deemed necessary, to be performed on a small subsample of S'tiếng males. Continued surveillance of malaria prevalence was particularly important in the population from which case control and cohort subjects were drawn.

Survey subjects and sites

The sample size was further reduced to 500 individuals per commune. A number of hamlets were excluded from this and subsequent surveys, in order to prevent the hamlet sample size from becoming too small. Eight hamlets in Đức Hạnh and 5 hamlets in Đa Kia were removed from the sampling frame. Two hamlets in Đức Hạnh which were participating in the cohort study but had hitherto been excluded from the surveys were added. No hamlets were removed in Đắc Ơ, although attendance remained negligible in Đak Lim, and none of the invited families in a further 3 hamlets attended this survey only. 200 unrelated S'tiếng males were selected at random for the G6PD assay.

Survey conduct

The survey was conducted between the 11th December 2001 and 11th January 2002 (no survey had been possible in August 2001 as all the efforts of the study team were focused on the initiation of the cohort study). The study form was modified slightly to elicit a history of haemoglobinuria (appendix 1). Survey conduct was similar to previous surveys. Individuals selected for the G6PD assay also had a 75µl haematocrit tube filled during capillary blood sampling.

Sample handling

Samples for G6PD analysis were kept at room temperature and transported to the district health centre within 4 hours of being taken. Other samples were kept on wet ice in the field, transported to HTD where they were kept at 4°C for 1-2 weeks whilst being processed as for samples in the fourth survey, thence stored at -20°C. Haemolysates were sent to Oxford in tranches in July 2002, March 2003 and May 2003.

Sixth survey

Aims

This survey was undertaken purely to monitor malaria prevalence.

Survey subjects and sites

The survey was conducted in the same hamlets as the fifth survey. The reduction in sample size and the decreasing success in encouraging whole households to attend prompted a change in sampling strategy to invite individuals rather than families, but with a strong emphasis placed on achieving a representative sample. Sample sizes were again constructed by ethnic group, age group and sex, and individuals selected locally and invited to attend.

Survey conduct

The survey was carried out between 10th and 24th April 2002. Two changes were made to the study form: the rewording of the bednet question to specify use the previous night, and the inclusion of a question on whether the subject had ever been admitted to hospital (appendix 1). The study conduct was similar to previous surveys.

Sample handling

Samples were kept on wet ice in the field, transported to HTD and kept at 4°C for 4-6 weeks whilst being processed as for samples in the 4th survey, thence stored at -20°C.

Seventh survey

Aims

HPLC analysis on samples from previous surveys had produced a few unusual results, in particular some unexpectedly low levels of HbA2. It had been postulated that these might be due to iron deficiency in those subjects. This survey aimed to quantify iron stores in a subsample of S'tiếng subjects in addition to continuing to monitor the prevalence of malaria.

Survey subjects and sites

The survey was carried out between 8th and 25th August 2002 in the same hamlets and utilising the same sampling methods as the sixth survey.

Survey conduct

General study conduct was similar to previous surveys with minor modifications to the form to enquire about breastfeeding and weaning for child participants (appendix 1). Capillary blood was collected into lithium heparin tubes in contrast to all other surveys.

Sample handling

Capillary blood samples were stored at room temperature prior to transport to the commune health station where they were spun, separated, aliquoted and frozen within 4 hours of being taken. Some of the haemolysates were sent to Oxford in May 2003.

Eighth survey

Aims

Documenting red cell indices amongst a sample of the study population had long been a project goal. By the end of 2002 it was deemed feasible after a pilot study with pregnant women in the cohort study. The opportunity of this survey, necessary to monitor the prevalence of malaria in the study district, was thus taken to characterise the

local red cell phenotype, and establish an appropriate multiplier for parasite counts recorded per WBC.

Survey subjects and sites

The survey was carried out between 4th and 19th December 2002 in the same hamlets and utilising the same sampling methods as the seventh survey.

Survey conduct

General study conduct was similar to previous surveys with minor modifications to the form to enquire about symptomatic anaemia (appendix 1).

Sample handling

The EDTA samples were kept at room temperature and transported to HTD the day that they were taken and analysed on a coulter counter programme giving values for haemoglobin, red cell count, red cell indices, total white blood cell count (but not a differential) and platelet count. Any remaining sample was stored at 4°C for between one and three weeks, before being separated and stored at -20°C. Haemolysates were sent to Oxford in May 2003.

Ninth survey

Aims

It had become apparent that a number of shipments of haemolysates were degraded by the time they arrived in Oxford. In addition to continuing to monitor malaria prevalence, this survey aimed to optimise the handling of samples for HPLC analysis.

Survey subjects and sites

This survey was carried out in the same hamlets as the eighth survey, using the same sampling methods.

Survey conduct

The survey was carried out between 9th and 24th April 2003. The conduct of this survey was similar to previous surveys. The survey tried to ascertain how many bednets each family possessed (appendix 1).

Sample handling

EDTA specimens were kept on wet ice for a maximum of 4 hours prior to transport to the commune health station, where they were spun, separated and aliquoted and stored on dry ice pending transport to HTD at the end of the week's survey. They were subsequently stored at -80°C.

Tenth survey

Aims

Despite continued efforts to improve the validity of the sampling methods, concerns that the selection of individuals was non-random persisted. This survey attempted to use the census lists to randomly select individuals automatically.

Survey subjects and sites

The KAP was planned to run around the time of this survey. It was felt that running both surveys in a particular hamlet would lead to poor co-operation with one or the other, so those hamlets included in the KAP in Đức Hạnh (Đak Khâu, Bù Gia Phúc 1, Bù Gia Phúc 2, and Thác Dài) and Đa Kia (Bình Giải and Bù Tam) were excluded from the survey. The situation was slightly different in Đắc Ơ, where the KAP was conducted as part of an ongoing longitudinal behavioural study, so all hamlets were included in this survey. A list of subjects to be invited was generated by selecting the required number of individuals from the top of randomly ordered lists filtered by hamlet, age group and ethnic group (S'tiếng or not S'tiếng). The required number of subjects in each group was generated from currently available population data assuming 500 individuals per commune and a representative sample. Hamlets excluded from the

survey were eliminated from the list after this process, resulting in a smaller sample size from those communes.

Survey conduct

The survey was carried out between 19th August and 5th September 2003. The conduct of this survey was similar to previous surveys. The survey tried to ascertain how many bednets each family possessed (appendix 1).

Sample handling

Sample handling was identical to the ninth survey.

Case control design

The incidence of severe malaria is low even in high transmission regions, requiring a cohort of many thousand susceptible individuals in order to demonstrate even a large protective effect. In low transmission regions the cohort sample size becomes even less feasible. It may increase further if the cohort is under close observation, as the ethical requirement to treat any detected uncomplicated malaria will reduce the incidence of severe disease (Hayes et al. 1992). Case control designs offer a practicable solution by utilising the almost inevitable contact with health care services of individuals suffering from severe malaria to sample affected individuals regardless of the population from which they are drawn. Constructing a panel of unaffected controls representative of the population from which the cases are drawn then allows comparison of risk factors for severe malaria. Case control studies are fraught with methodological pitfalls, however, and are more likely to result in a biased estimate of the effect under examination than other possible designs [illegible].

Issues of bias are often considered in categories of information bias, selection bias and confounding. Information bias may affect measurement of exposure, determination of case status, or measurement of other variables. A particular type of information bias

resulting from the incorrectly assigning exposure or status is known as misclassification bias. Information bias will act as a negative bias (ie the estimated OR will be closer to 1 than the true OR) unless the magnitude of error is different in cases and controls, in which case the bias may act in any direction. Differential recall bias in studies of risk factors for cancer leading to overestimation of the effect of certain exposures is a frequently cited example of a positive information bias. Misclassification is always a negative bias.

Selection bias is a particular problem for case control designs, as many possible control groups are not strictly comparable to the cases. Controls should have the same "base experience" as cases (Miettinen 1985). This concept encapsulates the principles that controls should be drawn from the same population (defined in space and time) as the cases, that they were at risk of becoming cases, and they would have become cases in the study were they to have developed the condition under examination. There are two approaches to meeting these criteria: in order to use randomly selected population controls it is essential that case ascertainment is complete (Miettinen 1985; Savitz et al. 1988). This may be achievable in some circumstances (e.g. a meticulously kept national disease database for a rare condition), but is manifestly impossible in others. Severe malaria is one such condition, as individuals may die before reaching hospital or present to centres other than those recruiting. In these situations controls must be selected from the "secondary base" - the population (defined by nebulous factors such as access to health care as well as geographically) from which the cases are *actually* drawn. Defining this base precisely is essentially impossible, and a number of different strategies have been used to approximate it. Two of the most frequently used are matching of controls to cases, and selecting controls from a limited population which shares some feature of the cases (eg using hospital inpatients as controls for cases recruited at hospital admission). Controls may be frequency matched by randomly selecting a number of individuals from each stratum of the population to correspond to

the number of cases in that stratum, or individually matched, whereby one or more controls with the same set of predetermined factors as the case are selected from the population (Wacholder et al. 1992b). Prospectively gathered, individually matched controls are likely to represent the best approximation to the secondary base, but have their disadvantages. Most prominent amongst these is the risk of over-matching: the use of matching criteria which are either very specific or numerous can effectively lead to matching on exposure, resulting in a profound negative bias (Choi et al. 1984).

Confounding arises where an independent risk factor for the case defining condition, which does not lie on the causal pathway between the exposure and the disease under study, is associated with exposure in the study population. It is not restricted to case control studies, but may be ameliorated by careful control selection.

Although not a cause of bias, a final important consideration in the design of case control studies is efficiency (Howe et al. 1983; Wacholder et al. 1992a). Studies will vary as to whether cases or controls are more difficult to recruit, and the study design should aim to minimise loss of information from scarcer subjects. Such loss might occur if the distribution of an important confounder was markedly different in cases and controls, for instance. If ethnicity was related to disease, for example, there might be 100 subjects of one ethnic group in a total of 300 cases, but a random population sample of 300 controls might only contain 30 individuals of that ethnicity. The optimum ratio of controls to cases will usually vary between 2:1 and 1:2, depending on relative ease of recruitment, as statistical efficiency deteriorates at more extreme ratios (Ury 1975). Recruitment of cases without controls or vice versa has an obvious negative impact on efficiency, as will any misclassification, even if detected prior to analysis. The discovery of an unexpected confounder will clearly affect the power of the study. This finding might sometimes be interesting in and of itself, but is often a local epiphenomenon, and would have best been avoided.

The recruitment of sufficient cases of severe malaria was always likely to be the limiting factor in this study environment, thus an appropriate and reliable case definition was necessary both to minimise misclassification bias and maximise efficiency. Attributing a disease process to malaria, and defining the disease as severe are both necessary in establishing that an individual is suffering from severe malaria, and neither is straightforward. The lack of comparability between both research publications on severe malaria and public health reports of severe malaria incidence prompted the establishment of an expert committee under the auspices of the WHO with the remit of generating a generally applicable, watertight definition (Beales et al. 1990). The criteria laid down in the committee's report are summarised in table 2.3 (overleaf). Whilst a significant step forward, this report has not gone unchallenged, and although severe malaria studies are less disparate than they were, there have been very few such investigations since the publication of this document which have not modified the WHO criteria in some way. This is, perhaps, unsurprising, given the variation in epidemiology and clinical presentation of severe malaria between regions.

The issue of asymptomatic parasitaemia was raised in chapter 1. The existence of malaria parasitaemia on a peripheral blood film in an individual with a clinical syndrome compatible with severe malaria is subject to the same caveats as this finding in an individual with fever alone. The potential differential diagnoses in the case of mild malaria are common and difficult to establish with certainty, whereas the differentials in severe malaria are either rare, or easy to distinguish with the assistance of unsophisticated investigations or through response to treatment. There are exceptions to this, such as a "mild" encephalitis resembling cerebral malaria, but most investigators have accepted any asexual parasitaemia in the face of a severe malaria syndrome with no other cause as being a reasonably solid case definition. Difficulties may arise in fatal cases, as response to treatment is unhelpful, and some recent work has suggested that causes of death in children apparently suffering from severe malaria are

varied (Taylor et al. 2004). Fortunately death from severe malaria is rare in the study environment, and as most of the alternative causes of death identified in the Malawi study would be almost certain to result in mortality if left untreated, response to treatment provides further confirmation that the clinical syndrome was due to malaria.

Criterion	Definition
Cerebral malaria	Unrousable coma persisting for >30 minutes after last convulsion
Repeated convulsions	>2 witnessed convulsions in 24 hours despite cooling
Severe anaemia	Hb<5g/dl or Hct<15% with parasitaemia >=10,000/µl and normochromic erythrocytes
Renal failure	Urine output <400ml in 24 hours (or <12ml/kg/24hrs in children) failing to respond to rehydration AND creatinine>265µmol/l
Pulmonary oedema or ARDS	Not specifically defined
Hypoglycaemia	Blood glucose <2.2mmol/l
Shock	Systolic blood pressure <70mmHg in adults or <50mmHg in children aged 1-5 years with signs of poor peripheral perfusion
Spontaneous bleeding	Any non-traumatic bleeding or laboratory evidence of disseminated intravascular coagulation
Acidosis	Arterial pH<7.25 or plasma bicarbonate <15mmol/l
Macroscopic haemoglobinuria	Specifically excludes drug induced haemolysis in individuals with G6PD deficiency
Manifestations which may define severe malaria in certain circumstances	
Impaired consciousness	Not satisfying criteria for unrousable coma
Prostration	Inability to sit or walk without obvious neurological cause
Hyperparasitaemia	Not defined
Jaundice	Clinically apparent or serum bilirubin >50µmol/l
Hyperpyrexia	Rectal temperature>40°C

Table 2.3 WHO criteria for severe malaria

Case definition in this study was further complicated by consideration of the degree of severity of malaria at which HbE might have its effect. It is not necessary for HbE to protect against direct malaria mortality in order to increase fitness. Reduction in morbidity is likely to have had a significant impact on the ability to thrive in a competitive environment. Protection against mild malaria, much more frequent than severe disease, might well be the most important. Whilst that question was beyond the scope of this study (a planned mild malaria case control study proved impossible to

organise), the severity criteria were deliberately lax in order to capture an effect of HbE on complicated malaria. Care was taken to ensure that the criteria would define a subpopulation of inpatients, however, and not overlap with outpatient cases, to avoid bias due to differential admission of certain groups (see discussion of hospital controls below).

Assigning HbE genotype is, fortunately, a very robust procedure, so information bias is not a significant concern with regard to exposure.

Death from malaria without contact with health services is rare in the study community. The cases were drawn from a much larger population than that which was studied in detail, however, and any extrapolation might be invalid, although the northern communes of Phước Long are amongst the poorest and least accessible in the province and are likely to represent the nadir of health care utilisation. There is no doubt, however, that cases recruited at HTD and Đồng Xoài are drawn from a population which has access to other avenues of health care: the district health centres in the other three districts of the province, or Bình Dương provincial hospital to the south. Case ascertainment would therefore be insufficiently complete to use randomly selected population controls. Control groups considered were hospital controls, clinic controls and matched community controls. Whilst hospital controls are very likely to ensure that controls would have been cases if they had developed severe malaria, they may differ from the underlying (secondary) base in a number of ways. Inpatients are more likely to be suffering from other comorbidity, which is likely to have a different effect on risk of admission for different diseases or infections. Randomly selected inpatients, even if restricted to a group under interest such as those admitted for infections, will contain a higher proportion of individuals suffering from common conditions, and any risk factors associated with these conditions will be over-represented. It has been suggested that some genetic traits are linked to hospital admission, and although these might affect the

risk of admission for severe malaria in a similar way to that for other infections, they will be over-represented amongst hospital controls. The finding that certain haemoglobinopathies such as α thalassaemia (Allen et al. 1997) and HbS (Colombo et al. 1985; Le Hesran et al. 1999) are associated with a reduced risk of admission for non-malarial infections and all causes respectively is of particular concern. Two studies examining this phenomenon for HbE have been of poor quality and given conflicting results (Na-Nakorn et al. 1956; Sicard et al. 1979). After spending some time at Phước Long District Health Centre it became clear that reasons for admission frequently went beyond clinical need. Time of day, available transport, social circumstances and financial situation would often play a role, together with inevitable inter-doctor variability. This might often lead to different admission rates amongst different groups: a lower threshold for admission for poorer ethnic minority groups (with, in the case of the S'tiếng, a much higher prevalence of HbE) was likely, particularly at night, although to some extent militated against by ability to pay. Siblings of children requiring admission from families without transport might be admitted with minor illnesses if only one parent had attended with all the children. Using hospital admission as an indicator for eligibility as a control thus gave rise to concerns over the possibility of Berkson's admission rate bias, but generating criteria for the recruitment of hospital controls was thought to be difficult, as it would need to encompass a wide range of different conditions. In the absence of a dedicated study doctor in Phước Long, hospital controls were felt to be impracticable as well as theoretically suspect. Clinic controls were dismissed for broadly similar reasons.

Sufficient subjects would be involved in the cross sectional surveys to provide a population from which to frequency match controls. They were drawn from a limited geographic subpopulation of that which would supply the cases, however, and may not have been a random selection of the population. It was felt that prospectively gathered, individually matched controls would provide a much better approximation to the

secondary base. Key findings from the initial survey which informed the choice of matching factors were the apparent microheterogeneity of malaria transmission and the disparate prevalences of both malaria and HbE amongst the ethnic groups. Matching on location of residence would help control for malaria exposure as well as access to healthcare and a number of other poorly defined but geographically determined factors. Ethnic group was clearly a major confounder, and although possible to control for in the analysis, consideration of efficiency dictated that it should be a matching factor. The contribution of matching to efficiency has been questioned, with some authors arguing that matched designs are almost always less efficient (Choi et al. 1984), but the effects of ethnic group seem so significant that only a stratified analysis would satisfy confounding concerns, and this would be less powerful if appropriate numbers of controls were not present in each stratum. Age and sex were also chosen as matching factors, the former in case of differential mortality from any of the effects of HbE, the latter as a recognised potent modifier of biological interactions. The need to match on age and sex may be questionable in terms of satisfying the secondary base principle or confounding, but both were also felt to be important in avoiding the possibility of the control group being highly skewed in terms of age and sex due to sections of the population being away from their houses when control gathering teams called, or control team preferences (eg not wanting to try and bleed children). Other potentially important factors such as migration history, previous malaria exposure, bednet use and other individual behavioural risk factors were not considered for matching on the grounds that any association with HbE status would be unlikely, and the greatly increased risk of overmatching if these factors were used.

Associations between genetic traits (in this case haemoglobin E) and a potential phenotypic association (in this case a negative association with severe malaria) are also amenable to examination by family based studies: given the genotypes of cases and both parents, the frequency of transmission of alleles from parent to offspring is compared

with that expected under the null hypothesis that both alleles will be transmitted with equal frequency. This is examined statistically with the transmission disequilibrium test (TDT) (Ewens et al. 1995; Spielman et al. 1996). The TDT has been extended to use data from siblings (Knapp 1999; Schaid et al. 1997), although this is not without its pitfalls (Cervino et al. 2000; Curtis et al. 1995; Curtis 1997). A recent review has found this method to be useful and reliable in estimates of the protective effect of HbS against malaria, and proposed just the sort of design adopted in this study (Ackerman et al. 2005).

Sample size and study sites

Sample size calculations were based on the community control design alone, as it was impossible to estimate in advance how many families we would successfully recruit. Based on the assumptions of a 50% protective effect of homozygous HbE, 25% protection from heterozygous HbE, and a gene frequency of HbE of 0.1 in the base population, the required sample size for a 1:2 case:control study design was 450 cases. The assumed gene frequency was postulated on the basis of an estimated 30% of cases being from the S'Tiếng ethnic group, approximately half of whom carry HbE, and minimal HbE in other ethnic groups. We planned to carry out the study over a 3 year period. Recent experience at HTD and admission records for Phước Long suggested that we could expect to recruit approximately 50 patients per centre per year. The third centre required to attain the required sample size was Bình Phước provincial hospital in Đồng Xoài, the provincial capital, which lies on the referral pathway between Phước Long and HTD. This hospital acts as a primary and secondary centre for the residents of Đồng Phú district, although patients may be referred or choose to attend from health stations outside this nominal catchment area. The hospital is the tertiary referral centre for the district hospitals in Phước Long, Bù Đăng, Bình Long and Lộc Ninh, although more seriously ill patients may be sent directly to the larger hospital in the capital of Bình Dương province to the south, or the relevant speciality hospital in HCMC.

Individuals with severe malaria, for instance, are likely to be referred to Bình Phước if they have severe anaemia requiring transfusion (or moderate anaemia in a pregnant woman), cerebral malaria not requiring ventilation but not improving after about 24 hours treatment, or, occasionally, complicated malaria and poverty, as the provincial hospital has a larger budget for subsidising health costs for these individuals. Acidotic patients, those with several complications and those requiring or likely to require ventilation or renal replacement therapy, are likely to be referred directly to HTD.

The study commenced in June 2000 at two centres: Phước Long District Hospital and the Hospital for Tropical Diseases, HCMC. The extension to Đồng Xoài was delayed by the fallout from the ethnic minority unrest in Đắc Lắc, then by repeated postponements of the move to new premises. Although 10 cases were recruited in October 2001, during a brief period of residence by Dr Thái (one of the study doctors from HCMC), consistent recruitment did not begin until December 2002. The relatively slow rate of accrual of S'tiếng patients prompted overtures to a fourth centre, the district hospital of Bù Đăng district, in mid 2001. A commune of Bù Đăng had been included in the first survey, and in addition to demonstrating a moderate prevalence of malaria, the population comprised significant numbers of M'Nong ethnic minority people, who demonstrated a prevalence of HbE of 35%. Political protocol considerations delayed recruitment in Bù Đăng until July 2003, by which time the incidence of severe malaria was so low that only 1 case was entered into the study in the ensuing year, and this centre was dropped in June 2004.

Case definition

Entry criteria were based on the WHO definition of severe malaria, expanded to include poor feeding in infants (as a surrogate for prostration) and respiratory distress. As discussed above, the criteria were relaxed in order not to miss an effect of HbE on complicated malaria. Individuals with jaundice alone, any impairment of consciousness

or parasitaemia>100,000/μl were included. The parasitaemia cut off was chosen for two reasons: admission levels of parasitaemia associated with a poor outcome might be expected to be lower in environments where artemisinin derivatives, capable of delivering dramatic decreases in peripheral parasite counts after just a few hours treatment, are freely available in the community; and a study in an epidemiologically similar region in northeastern Thailand had found an increased mortality associated with this level of parasitaemia. The final set of qualifying clinical features are presented in table 2.4. It was important to ensure that cases meeting more stringent definitions of severity could be analysed separately, so sufficient clinical data were collected on all cases to allow their classification as severe or moderate according to the criteria laid out in table 2.5, which had been used in treatment trials of severe malaria conducted locally. The entry criteria varied slightly between centres, as it was not possible to install the capability to measure bicarbonate in Phước Long, Bù Đăng or Đồng Xoài, and the prostration surrogate of poor feeding in babies was irrelevant in the predominantly adult environment of HTD. In addition to meeting the clinical criteria, the study required patients seen at HTD to have an address in Bình Phước in order to be eligible. This might be a permanent or recent temporary address, and was necessary to restrict our community control gathering operation to a feasible geographical area.

Cases were recruited by the admitting doctor. The medical staff at HTD have considerable experience of conducting or being involved in clinical research. Those in Phước Long, Đồng Xoài and Bù Đăng have little or no such experience, however. For three months prior to and three months after the commencement of recruitment a doctor from HTD was resident in Phước Long to assist in making decisions about eligibility of patients to enter the study, inviting consent, completing the study forms and taking appropriate samples. A study doctor was also intermittently resident in Đồng Xoài in the early stages of recruitment. Three different study forms, requiring varying levels of detail, were used in the four centres (all are included in Appendix 1).

Criterion	Definition
Impaired consciousness	Paediatric coma score <=4 or GCS<12
Repeated convulsions	3 or more seizures in 24 hours despite cooling, at least one of which witnessed by health care professional
Severe anaemia	Hb<5g/dl or Hct<15%
Hyperlactataemia	Lactate>4mmol/l at 0 or 4 hours
Acidosis	HCO3<15mmol/l
Renal impairment	Urine output <400mls (adults) or 12mls/kg (children<15) per 24 hours or creatinine>3.0mg/dl (265micromol/l)
Hyperparasitaemia	>100,000 parasites/microlitre
Respiratory distress	Any of nasal flaring, intercostal/subcostal recession, use of accessory muscles, deep breathing
Poor feeding	Taking very little (<25% normal) milk feeds in infant <8months old
Macroscopic haemoglobinuria	Visual inspection of urine
Jaundice	Clinical
Shock	Cold peripheries and clinical impression of shock in young children. SBP<70mmHg in adults.
Spontaneous bleeding	Atraumatic bleeding from gums, nose, GIT, old wounds
Hypoglycaemia	Blood glucose <40mg/dl (2.2mmol/l)

Table 2.4: Entry criteria for case control study (one or more of above criteria and asexual forms of *Plasmodium falciparum* on blood film)

Criterion	Definition
Impaired consciousness	GCS <11
Repeated convulsions	3 or more seizures in 24 hours despite cooling, at least one of which witnessed by health care professional
Severe anaemia	Hct<20% AND Parasitaemia>100,000/μl
Hyperlactataemia	Lactate>4mmol/l
Acidosis	HCO3<15mmol/l
Renal impairment	Urine output <400mls per 24 hours or creatinine>250μmol/l)
Hyperparasitaemia	>10% parasitaemia
Macroscopic haemoglobinuria	Visual inspection of urine
Shock	SBP<80mmHg
Spontaneous bleeding	Atraumatic bleeding from gums, nose, GIT, old wounds
Hypoglycaemia	Blood glucose <40mg/dl (2.2mmol/l)

Table 2.5: AAV criteria

The different study forms primarily reflected the varying degree of research experience at the different centres. Whilst we wished to gather as much clinical data as possible in Phước Long and Bù Đăng, in order to ensure all cases were correctly attributed, this level of detail was neither necessary nor practicable in HTD, where different studies of

the treatment of severe malaria, requiring their own sets of study notes, were ongoing. The notes for Đồng Xoài represent a refinement of the Phước Long notes, taking into account the greater degree of specialisation of the staff in the provincial hospital.

Consent was sought by the recruiting doctor, as it was not feasible to have a study doctor available full time at each of the centres. A written information pack was compiled and translated into Vietnamese. This pack included a detailed but digestible description of the aims and methods of the study for the recruiting doctors to impart to study subjects. Training sessions were held at each of the remote hospitals for the doctors involved, and a lead clinician was appointed by the local hospital director at each peripheral centre. A draft patient information leaflet was produced, but, on the advice of our local collaborators, was not used. A simple written consent form, specifying that the study had been explained to the patient or family by the recruiting doctor and that consent was given was implemented in its stead. A similar form was used for controls. Treatment decisions in Vietnam are often made by the family, rather than the patient themselves. Written consent for this study was always sought from the patient, however, unless they were comatose, in which case consent was sought from accompanying relatives, or a child, in which case consent was sought from parents. The study budget provided for all malaria related treatment and food for the duration of the hospital stay in Phước Long, Bù Đăng and Đồng Xoài. These costs were already borne by the intervention study for the majority of cases recruited at HTD, but the study covered the costs of the few not severe enough to be eligible for the treatment study. Staff involved with the study at hospitals other than HTD were paid a monthly and a per case sum. All study notes were checked by one of the study doctors, and discrepancies resolved with original case notes where appropriate and possible. All blood smears were re-read by expert microscopists at HTD.

Community controls

The community controls were recruited by the local Malaria Control and Public Health team in Phước Long, by one of the study doctors in Bù Đăng, Lộc Ninh, and Bình Long, and by Dr Thái or the provincial malaria control team in Đồng Phu. Each team underwent training and 4 control collections with a member of the study team before undertaking control collection unsupervised.

Two controls were collected for each case. The team visited the address given by the case, confirmed that the case was living or had lived there, and enquired about the age and sex of occupants of nearby houses. Two individuals of the same ethnic group as the case but unrelated to the case or to each other were selected from one of these houses. Attempts were made to match controls with the sex of the case, and to recruit controls within the age bands detailed in the adjacent table 2.6. Written consent was sought from the individual or parent in the case of a child, and 250-1000µl of blood was taken from a finger prick using high volume lancets (FreeFlow, Beckton Dickinson) into a specialised EDTA containing collecting system (Greiner). Control subjects were offered vitamin supplements and, if the team included a doctor, a few days treatment for any coincidental infections.

Age of Case	Age of Control
<1	<1
1	1
2	2
3	3
4	3-5
5	4-6
6-14	Age +/- 1 year
15-20	Age +/- 2 years
20-60	Age +/- 5 years
60+	Age +/- 10 years

Table 2.6. Age matching bands

Family controls

A capillary blood sample was taken from both biological parents if they were present and consented. If only one parent was available we endeavoured to bleed this parent and as many siblings as possible in order to attempt to "reconstruct" the missing parent. The need for two family controls became engrained quite early in the study, however,

and we rarely obtained samples from more than one sibling. The study protocol also envisaged adding to the family controls during the community control gathering visit to the case's house, though this rarely transpired, and obtaining as many sibling controls as possible for adult patients, which was rarely possible.

Sample handling

Blood samples were kept at 4°C until transport to HTD for separation and aliquoting of the pellet. Pellet aliquots and plasma were frozen. One aliquot was transported frozen to Oxford for HPLC analysis of haemoglobin (as described in appendix 3), and one aliquot subsequently thawed for DNA extraction. The DNA was amplified using the Genomiphi whole genome amplification system (Amersham, details in appendix 3).

Blood cultures were taken on admission and incubated locally (in incubators provided by the study) for 1-5 days before transport to HTD. If a transport run was missed and the culture arrived in HTD 7 or more days after being taken, it was discarded.

Analysis methods

Matched case control comparisons were conducted by conditional logistic regression. This maximum likelihood method estimates the probability that the case carries the genotype (more generally the risk factors or dependent variables under study) actually found, given the genotypes of the case control set and that one member of the set is a case. This technique allows for more than one control per case, and can generate separate odds ratios for heterozygous and homozygous HbE. The chi squared statistic was used to establish significant differences in all 2x2 tables with independent variables, except where any cell contained less than ten observations, in which case Fisher's exact statistic was used. The transmission disequilibrium test (TDT) examined anomalies in transmission where triplets of case and both parents were available. All analyses were carried out using Stata 8.0 (StataCorp LP, Texas, USA).

KAP design

Aims

The knowledge, attitudes and practice (KAP) was conducted in order to confirm or refute local assertions about behavioural differences between the ethnic groups, and suggest or rule out these differences as potential explanations for the inter-ethnic variation in malaria prevalence.

Sampling methods

Hamlets in the three cross sectional survey communes of Đắc Ơ, Đức Hạnh and Đa Kia were chosen to participate in the study if official figures documented an ethnically heterogeneous population. Further confirmation was sought from the local health care and administrative staff, and hamlets excluded if the different ethnic groups were said to live in geographically distinct population centres within the hamlet. This process yielded 10 study hamlets: Thôn 4, Thôn 7 and Bù Bưng in Đắc Ơ, Bình Giải and Bù Tam in Đa Kia, and Đak Khâu, Bù Gia Phúc 1, Bù Gia Phúc 2, and Thác Dài in Đức Hạnh. Households were selected at random from the 2000 census list in Đa Kia and Đức Hạnh, and a freshly prepared household list in Đắc Ơ, where a longitudinal KAP study was planned as part of the entomological survey. The household lists were stratified by ethnic group prior to random selection. Only two strata, S'tiếng and non-S'tiếng, were possible in the hamlets in Đa Kia and Đức Hạnh, as ethnic group data was not collected in the 2000 census, and only the S'tiếng have sufficiently characteristic naming patterns to allow ethnic allocation on the basis of available data. Once the household sample list had been generated, further checks were made with the hamlet YTTB to confirm that those families were still resident in the hamlet. Families said to have moved away were replaced by randomly chosen families from the same ethnic category. These additional checks were not made in Đắc Ơ. Once the sample was

finalised, an additional 10 households in each ethnic category were randomly selected from the remainder to be used in case of failure to find one of the sample households.

Sample size calculation was almost impossible for a number of reasons: with the exception of bed net use, we had very little information on the proportion of households expected to exhibit any particular behaviour; the questionnaire design included elements for both individuals and households, raising the issue of how the sample size should be determined; a number of different classes and elements of information were being gathered simultaneously, possibly necessitating adjustment for multiple comparisons (although whether this would, in fact, be required, was moot). There was a practical absolute upper limit on the numbers as some of the hamlets were quite small. In the end a pragmatic 600 households (roughly 70 per hamlet), with an approximate 40:40:20 split between Kinh, S'tiếng and Tày-Nùng, was chosen to give reasonable confidence intervals on estimates of behaviour prevalence in any one ethnic group. This sample size turns out to be capable of resolving differences of 10-15% for prevalences around 50%, and being sufficient to measure a difference of 8% in bed net use from the expected 99% amongst the Kinh.

Questionnaire design

The questionnaire was designed in collaboration with staff from the Institute of Malariology, Parasitology and Entomology and Dr Mary Chambers, also working in the Wellcome Unit in Hồ Chí Minh City. The questionnaire was divided into three sections: the first, to be completed by the most senior member of the house available, concerned issues pertinent to the household as a whole, such as annual income, crops grown, and number and last treatment of bed nets. This section included some questions on general household behaviour such as the usual bed times and health care seeking behaviour of adults and children in the family, as well as space for the interviewer to note the condition of bed nets, the distances to forest and stream,

the materials used in house construction and to score the "openness" of the house. The second section was a table of all the family members in which to record the names, ages, ethnic groups and relationships of all individuals in the household, together with some information on migration, bed net use, and forest visits for each member. We hoped that this structure would encourage the respondent to be questioned about the behaviour of each family member in turn, avoiding the inaccuracy inherent in questions such as "Does anyone in the family go and work in the forest?", providing finer granularity and discovering which ages of children, if any, usually accompanied one or other parent on trips to the forest. This structure also allowed us to check the accuracy of our 2000 census data for that household. The third section was an individual questionnaire, to be administered to two family members. In addition to confirming information such as bed time, bed net use, forest work and use of bed net when sleeping away from home, this contained a few questions on educational achievement and knowledge of malaria transmission and prevention. This section thus acted as a validity check for the family members table and as well as extending the information on that individual. The resultant, somewhat cumbersome, design was deemed necessary for two reasons: a questionnaire aimed solely at individuals would be likely to miss the working males in the family, who would not be at home when the interviewer visited, and would also gather no information on children's behaviour. The results would thus lack data from two of the three groups at greatest risk for malaria (pregnant women being the third). A questionnaire which gathered only general information about the household, however, would miss the opportunity to discover how individuals actually behaved, particularly when away from the home.

Different approaches to assessing the families economic status were felt necessary. There are incentives for families to both exaggerate and underestimate their annual income. There was a particular concern that the S'tiêng would identify the interviewers as agents of authority, and minimise their income in order to qualify for more

government aid. Thus we elected to ask about land ownership, cultivation of cash or purely subsistence crops, ownership of key consumer items, such as televisions or motorbikes, and rice purchasing patterns, which have been used successfully to quantify household income in other surveys in Vietnam.

Study conduct

The study was carried out by Dr Hưng from NIMPE in Đắc Ơ, and by teams from IMPE, HCMC in Đức Hạnh and Đa Kia. Consent in the KAP study was informal, the teams enquiring of the family whether they were happy to participate. The interviewing team visited the house, and administered the questionnaires to the head of the household if available, or the most senior person in the house if they were not. If they judged that nobody in the house would be able to accurately complete the household questionnaire, they returned to the house later in the week, if possible, or chose a house from the backup list. Blood smears were taken from two individuals per house, preferably including a child under fifteen.

Analysis

The main thrust of the KAP was to document any differences in behaviour between ethnic groups that might explain the variation in malaria prevalence. These associations were to be examined using standard methods (see data analysis section below). Two issues considered at the design stage were whether to correct for multiple comparisons, and how to treat individuals within a family, as there is obviously a lack of independence. No specific analysis plan was devised to encompass these problems in advance.

A secondary aim of the study was to examine which factors appeared to be high risk in this environment. Whilst some data would come from the blood smears taken as part of the study, we hoped to utilise data from previous contact with the families during the cross sectional surveys to bolster our findings. This became a more pressing

consideration when the prevalence of malaria in this study was found to be extremely low. This proved a theoretically taxing problem. Households had been chosen for inclusion in the KAP with no regard to previous contact, so individuals and families will have only participated in one, or possibly two, of the 8 surveys for which we have reasonable identification data. Thus, with the exception of a handful of households, we have nothing like the de facto cohort which would allow relatively straightforward quantification of malaria susceptibility. The obvious choice of indicator would be a simple binary variable reflecting whether an individual, or any individual in a household, had ever been smear positive. This has additional appeal in that the great majority of individuals would be expected to be smear negative, so the loss of information by effectively discarding discordant or multiple negative smears would be small. The problem with this approach is the significant variation in smear positive prevalence between surveys, risking the likelihood of an individual or family having been smear positive being a function of which survey they attended rather than any associated risk factors. This might not be a problem if individuals and families attending different surveys did not differ in any of our primary outcome variables, but this cannot be assumed (and in fact proved false). A further complication at the household level is the difference in age composition (and hence malaria risk) between households, and between household samples.

A number of different strategies to combine smear data from different surveys were considered, the advantages and disadvantages of which are displayed in table 1, appendix 2.

A particular difficulty common to all methods, especially at the household level, is which survey specific smear positive probabilities should be used as correcting factors. The possibilities are overall SPP in each survey, age group specific SPP in each survey, ethnic group specific SPP in each survey, and age and ethnic group specific SPP in each

survey. In trying to adjust for the secular trend towards decreasing malaria prevalence without adjusting for factors, such as age and ethnic group, which might be related to behaviour, the first of these options is appropriate. The difficulties entailed by the varying age composition of family samples prompted us to consider using age specific SPP's for the family calculations. These vary considerably by ethnic group, however (table 2.7), and the overall curve is almost flat, although the oldest family members uniformly have less malaria. Thus adequate age correction would also introduce an ethnic group related adjustment factor, which we wished to avoid. On balance the overall smear positive prevalence in that survey appeared to be the most appropriate adjusting factor.

Age group	Kinh	S'tiêng	Tày	Nùng	Other
<1	0	14 (8.0%)	1 (50.0%)	0	0
1	1 (0.7%)	23 (10.3%)	0	0	0
2-4	8 (1.3%)	170 (18.1%)	1 (2.6%)	1 (1.8%)	0
5-9	9 (0.9%)	343 (22.4%)	1 (1.4%)	2 (2.6%)	1 (4.5%)
10-14	11 (1.3%)	202 (19.6%)	2 (5.3%)	2 (5.0%)	0
15-19	19 (4.2%)	142 (20.0%)	2 (9.1%)	6 (13.0%)	1 (20.0%)
20-29	36 (4.0%)	171 (12.1%)	10 (11.6%)	17 (17.3%)	2 (10.5%)
30-39	25 (2.6%)	82 (7.6%)	8 (11.6%)	8 (12.1%)	4 (18.2%)
40-49	20 (3.0%)	79 (10.8%)	2 (5.0%)	1 (2.9%)	0
50-59	3 (1.0%)	37 (6.6%)	4 (18.2%)	5 (15.2%)	0
60-69	2 (1.2%)	21 (5.4%)	0	1 (7.1%)	0
70+	0	11 (5.1%)	0	0	0

Table 2.7. Number (percentage) smear positive by age group in the different ethnic groups

The final indices chosen were crude smear positivity status for households and individuals, checking that any significant results were not due to a survey effect and that any borderline results were not being underestimated because of a survey effect, and thirdly a calculated "smearness" index for individuals which took the value of the product of the probabilities of being smear negative in each survey which had included that subject if the individual had always been smear negative, and one minus this value if he or she had ever been smear positive.

Data handling

Data from all studies were entered into Microsoft Access databases, for the most part designed and coded by the author. Data was not double entered, due to resource constraints, but extensive validation routines were included to minimise gross errors at the time of data entry. Data were analysed using Stata 7 and subsequently Stata 8 (Timberlake Consulting). Charts were prepared using Microsoft Excel, modified where necessary in Corel Draw 9. Some maps were created using Health Mapper 2 (WHO).

Data analysis – general considerations

Assumption free tests were used wherever possible. The methods used for univariate analyses are presented in table 2.8. Multiple analyses were conducted by multiple logistic regression where the outcome was binary. As most of the associations of interest involved a dichotomy in the dependent variable, this was the most frequently used model. Categorical independent variables were expanded prior to inclusion. Parasitaemia was log transformed before inclusion in any model, but all other variables were native. Ethnic group was the major confounder in most analyses, and many associations were explored by calculating Mantel-Haenzel (ethnically) adjusted odds-ratios alone, rather than constructing more complicated models. Analysis of family triads was conducted with the transmission disequilibrium test.

Type of comparison	Visualisation	Screening test	Definitive test
Binary vs Binary	2x2 table	Chi squared (no visual confirmation)	Chi squared
Binary vs Ordinal	Nx2 table	Chi squared test for heterogeneity. Occasionally used T test.	If clear breakpoint in distribution of binary var wrt ordinal var, use chi squared of dep var against binary variable constructed around that value. If trend apparent but χ^2 not significant, used non-parametric test for trend.
Binary vs Continuous	Tabulation of mean & SE	T test	T test
Binary vs Categorical	Nx2 table	Chi squared test for heterogeneity	If one group stands out from the remainder, simple chi squared on dependent var and new binary independent var of that group vs the remainder, if clear dichotomy between groups of values, simple chi squared on dependent var and new binary independent var across dichotomy, otherwise chi squared test for heterogeneity.
Continuous vs Ordinal	As continuous vs categorical	Visual inspection and ANOVA. Occasionally used linear regression.	As continuous vs categorical, but binary variable for chi squared can only be constructed to separate values above and below a breakpoint, rather than grouping non-contiguous categories.
Continuous vs Continuous	Scatter plot	Linear regression	Linear regression
Continuous vs Categorical	Tabulation of mean & SE of continuous var by ordinal var, table of differences of means +/- dot plot or box plot	Visual inspection and ANOVA	ANOVA with Bonferroni and Tukey-Kramer post test of significance of pairwise differences. If there is a clear difference in the distribution of the continuous variable between one value, or a group of values, of the categorical variable and the other values, create a binary variable encapsulating this difference and perform T test.
Categorical vs Ordinal	NxN table	Visual inspection, chi squared test for heterogeneity. Sometimes used ANOVA or Kruskal-Wallis to explore differences in magnitude of ordinal variable.	Analysis methods not anticipated, and most dependent on data. Important relationships resolved to binary variables and tested by chi squared. Unresolvable patterns assessed with Kruskal-Wallis.
Categorical vs Categorical	NxN table	Visual inspection of proportions in table alone.	Explore hypotheses generated by inspection through generation of binary variables and running chi squared.
Ordinal vs Ordinal	NxN table	As categorical vs ordinal, with the occasional addition of linear regression and plot of regression line on scatter plot to examine overall relationships.	Similar to categorical vs ordinal. If both variables satisfied parametric assumptions and data appeared linearly related, confirmed visual suspicions with linear regression.

Table 2.8. Basic analytical methods employed.

Specific difficulties

Political

Bình Phước is a relatively new province, formed in 1989 by the division of Sông Bé province into Bình Phước and Bình Dường, the former lying closer to HCMC. Bình Dường retained the capital of Sông Bé (Thủ Dầu Một) and has experienced rapid economic growth, predominantly based on foreign investment in free export zones, whilst Bình Phước has remained a predominantly rural economy and significantly poorer than its more established and possibly more forward-looking neighbour. In order to visit Phước Long district, where most of the studies were based, the Hospital for Tropical Disease was required to apply for permission for me in advance. I was initially not permitted to stay overnight in Phước Long, although was allowed to sleep in Đồng Xoài, the provincial capital. This permission was eventually granted towards the end of 2000, and planning, organising and supervising the studies became much easier. It even became relatively straightforward to visit Đắc Ơ, most of which lies within the 40km border strip which is under the jurisdiction of the military, rather than civilian, authorities. In January 2001, however, ethnic minority groups, complaining about loss of traditional lands to Kinh immigrants, rioted in the central province of Đắc Lắc. This unrest was attributed to the malign influence of foreign protestant missionaries organising the minority groups and fomenting dissatisfaction with the strenuous efforts of the Vietnamese government to provide additional financial benefits for highland peoples and encourage them to lead a more settled and civilised way of life. It was six months before I was allowed to visit even the hospital in Phước Long, and close to a year before I could stay overnight or visit the hamlets. I have been accompanied by a plain clothes officer from the department for the protection of foreigners of the public security police on every subsequent visit to the hamlets. A moratorium was placed on all negotiations surrounding the cohort study, which wasn't lifted for 14 months. Once the protocol for the cohort study had been agreed with

Phước Long Health Centre, we sought the agreement of the provincial health services, as we had with all studies, but, as the cohort was composed of ethnic minority women and children, we also sought the permission of the People's Committee of Bình Phước Province, the Health Services of Hồ Chí Minh City, the People's Committee of Hồ Chí Minh City, and even the Ministry of Health, although the study was allowed to commence pending this last permission. Six months into the cohort study, the People's Committee of Phước Long District objected to our recruitment of pregnant women with two or more living children, as by doing so we were supporting them to disobey the "one couple, two children" policy of Vietnam. Following extensive negotiations by our Vietnamese collaborators at the Hospital for Tropical Diseases, we were permitted to follow up children already recruited to the study, but from that time forward were restricted to recruiting women with less than two living children.

Identification

One of the more intractable problems faced throughout this programme was the definitive identification of individuals in the community. Despite compiling a census list towards the end of 2000, it remained difficult to assign a participant in the surveys to an individual in the list. The issuing of unique identifying documentation was both unfeasible and deemed by our Vietnamese collaborators to be likely to decrease participation in the studies as we would be perceived as wanting to control rather than wanting to help. The collection of identity card numbers was similarly rejected as allying ourselves too closely with the authorities. In common with most rural environments, the only way to identify a household within the smallest administrative unit is by the name of the head of the household. The only parameters we have to identify individuals within a household is by name and age. The difficulties encountered with these three forms of identification are outlined below.

Head of household

There was considerable variability in the head of household reported by different individuals in the same house. There were three main sources of this variation. The first was whether a wife or husband of the son or daughter living in the household with his or her parents would report the name of her or his father (or occasionally mother), her husband, or the actual head of the household. The second was an apparent difference in opinion as to who was the head of the household in the event of the death of the most senior male member if he was survived by his wife, and conversely who was the head of the household when a mother or father moved back in with her or his child. The third was the problem inherent in reporting any name described below.

Names

The difficulties encountered with the reporting of names differed between the ethnic groups. Amongst the Kinh, Tày and Nùng, the main issue was the variation in middle names and the interchangeable use of formal names and household or nicknames. There was also some variation in the transcription of tones, which posed both technical problems in making comparisons in the database, and identification of individuals within families with similar names but different tones, the most extreme example of which was three sisters named Thủy, Thùy and Thụy. Amongst the S'tiếng there were also problems in transcription of S'tiếng names into Vietnamese. S'tiếng is an oral language, with no written equivalent in current use. It is extremely different from Vietnamese, being atonal and much more consonant based. Thus there is no correct way to write a given set of S'tiếng sounds in Vietnamese. The name Zen, for instance, might be transcribed as Zen (despite the absence of Z in the Vietnamese alphabet), or possibly Zên, by a southern Vietnamese speaker, or as Den or even Gien (unusual) by a northern Vietnamese speaker (and again, the e could be written ê). With a flowery script, the D might be mistaken at data entry for a Đ. A final possibility would be Ghen. Another example is the name Úy,

which might be rendered as Uy, Wi, Quí, Quý or Wí, or, if misheard, as Quyền, and there is an additional layer of technical problems in that correctly the rising tone mark here should span both U and Y, so during data entry both Úy and Uý would be correct, but clearly different once stored electronically. The difference in pronunciation between northerners and southerners add additional layers of complexity to trying to generate a Soundex type of system for phonetic matching. In the south, D and GI and Y have a similar sound at the beginning of the word, whereas in the north they have a soft Z sound. Southern regional accents would add V to the list of Y sounds, and northern R to the list of Z sounds. Certain other letter combinations have a similar lack of phonetic specificity, and the degree of specificity would also depend on which position the letters occupied in the word. Any phonetic matching system which took account of all these possibilities would have a very low specificity when searching for names, and would thus not be useful.

I eventually settled on stripping away all diacritical marks and apostrophes, substituting certain letters with similar sounds or appearances (I for Y, S for X, D for Đ, D for GI followed by any vowel, B for V followed by any vowel other than I, and MA for M followed by any vowel other than I), eliminating the vowel in certain consonant-vowel pairs (S followed by A, E or O, any vowel following P, G or K, and any vowel other than E following B or D), and reducing CHO and CHA to CH, as being a reasonably sensitive and specific system. Additional difficulties with regard to identification were presented by the S'tiếng traditionally eschewing the use of surnames. When forced to take surnames by the authorities, all the men were named Điểu, and all the women Thị. Thị is also a common Kinh middle name denoting a women, and as some of the S'tiếng aspire to be closer to their richer, more powerful compatriots, certain women have adopted Điểu Thị as a compound surname. This is often used interchangeably with Thị by field workers interviewing these women.

I didn't appreciate many of these complexities for approximately a year into the programme, which entailed considerable retrospective analysis of the databases to assign internal identity numbers.

Ages

The imprecision with which ages were reported added to the difficulties in identification, and was a significant concern during age related analyses. Once again, this problem was particularly severe amongst the ethnic minority groups, especially the S'tiếng. Different age groups were affected to different extents. Amongst small children, the main concern was during analysis, as malaria relevant age groups are quite narrow here.

Laboratory methods

Blood film microscopy

Thick and thin smears were stained with Giemsa (appendix 3) and read by expert microscopists. The team of microscopists at HTD have many years experience of reading films for both clinical and research purposes, and are a great asset to the unit, providing a real "gold standard" result. The whole thick smear was examined before a slide was declared negative. Positive smears were speciated, and trophozoites counted per 400 white blood cells, or per 1000 red blood cells in the case of high parasitaemias. Parasite counts per microlitre were then calculated using correction factors of 200 if counted by WBC's or 4000 if counted by RBC's.

Haemoglobin HPLC

Haemoglobin typing was performed in the dedicated Variant system (Biorad) on the beta short programme. Haemoglobin of different types bound to a dedicated cation exchange column from a 1 in 100 dilution of haemolysate elutes at different times. A typical trace is shown in fig 2.2. This method can distinguish all the major abnormal haemoglobins due to mutations in the beta globin gene (HbC,D,E,S). Some elute in their own window, whilst

HbE elutes with A2. β-thalassaemia trait carriers usually have an elevated A2 in the range of 3.5-10% of the total, HbE heterozygotes appear to have HbA2's in the range of 20-40%, and HbE homozygotes >85%. Preliminary testing of various sample storage and handling conditions had not revealed any significant variation from expected values, although they were carried out on normal blood. Proportions of HbE in heterozygote and homozygote individuals in the survey were lower than expected, however, the former being between 15 and 39% (mean 27.1%) and the latter between 75 and 99% (mean 88.3%). Whilst these ranges were a little lower than expected, the peaks in distribution were significantly distinct that there was no possibility of misattributing the HbE genotype. β-thalassaemia upper and lower exclusive bounds were 3.5 and 10% respectively. Sequencing supported the use of these cut off values: of the 99 individuals falling into this category, 76 underwent sequencing of the common β-thalassaemia mutation regions, and 74 had a detectable mutation. HbF levels were higher than usual, especially in individuals with HbE (both homo and heterozygotes). The lab experience was that this phenomenon is probably due to degradation products eluting in the F window. The only impact this is likely to have on genotype assignment it to complicate the identification of HbE/β-thalassaemia compound heterozygotes.

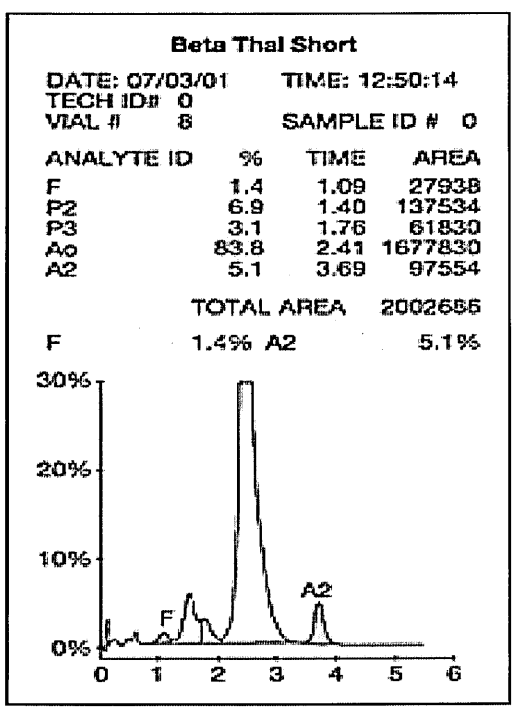


Fig 2.2. Typical output from the beta short programme on the Variant dedicated HPLC analyser

DNA extraction

DNA was extracted by two different methods. The first surveys were extracted by a whole blood phenol-chloroform method, detailed in appendix 3. This had given the best yields in the hands of the researchers in Prof. Clegg's lab in Oxford. Various methods were assessed by Vietnamese PhD students in the Wellcome unit at HTD, and no difference in yield or apparent purity of DNA, as measured by performance in difficult PCR assays, was found between the phenol-chloroform method and a spin column kit (Qiagen), although the resin based kit (Nucleon) did not seem to perform as well. In the interests of safety the Qiagen kit was thus adopted for DNA extractions in Vietnam. The second tranche of samples processed in Oxford for α-thalassaemia genotyping were also extracted using a kit.

Whole genome amplification

Two methods for maximising the number of assays that could be run on the DNA from the case control study were assessed: primer extension pre-amplification (PEP) and the newer multiple displacement amplification (MDA). The PEP product did not perform well in the alpha globin PCR's and was discarded, so will not be discussed further. MDA relies on the unique characteristics of a bacteriophage DNA polymerase named φ29 which shows long segment polymerisation with a strong strand displacement function (Blanco et al. 1989; Dean et al. 2001). Random hexamer primers anneal to template single stranded DNA and initiates replication at multiple sites. As synthesis proceeds, any given polymerase unit will reach the tail of another strand, and displace this strand to generate new single stranded DNA which can then act as a template for further polymerases. The strands are many kilobases in length (the enzyme has been demonstrated to polymerase single stranded DNA of 70kb (Blanco et al. 1989)), and are said to be an unbiased amplification of the genome (Hosono et al. 2003). Genomiphi kits (Amersham) were used to perform MDA in this project.

Alpha thalassaemia genotyping

The methods used for the detection of $\alpha^{3.7}$, α^{SEA} and $\alpha^{4.2}$ mutations are broadly those described by Liu and colleagues (Liu et al. 2000) and detailed in appendix 3. A significant modification undertaken in Vietnam was the substitution of a non-proof-reading hot start polymerase for the Amplitaq Gold in simple $\alpha^{3.7}$ & $\alpha^{4.2}$ reactions, with no apparent negative impact on reliability. There was greater variability in the assay for Hb_{CS}, although all methods were based on the technique of Viprakasit for distinguishing Hb_{CS} from Hb_{Pakse}, a mutation at the same site, detailed in appendix 3 (Viprakasit et al. 2002). Once it became clear that Hb_{Pakse} was uncommon in the study populations, an ARMS PCR for Hb_{CS} was developed to try and reduce the (restriction enzyme associated) costs. These results are not presented in this thesis, however, so this method is not described in detail.

G6PD assays

Rapid test

The rapid test was devised by Hirono and colleagues and has been extensively validated in the region. The test relies on the reduction of the tetrazolium dye 3(4,5 dimethylthiazolyl 1-2)2,5 diphenyltetrazolium bromide (MTT) to blue formazan in the presence of a hydrogen carrier (in this case phenazine methosulphate) by NADPH produced by G6PD. Haemoglobin reacts directly with MTT producing a dark red discolouration which can interfere with reading the test, so the G6PD in blood is absorbed onto diethylaminoethyl-Sephadex A50 (DEAE-Sephadex) and the reaction proceeds only within the gel layer at the bottom of the tube. Details of reagents are given in Appendix 3, but substrate and dye mixtures were, in fact, supplied prepared by Prof Matsuoka of Jichii University. The mixtures were aliquoted into 1.5ml eppendorf tubes at HTD or in the field, up to 24 hours before the tests were conducted.

Quantitative

The quantitative assay was conducted with a commercial kit (Sigma) which relies on the different absorption of NADP and NADPH at 340nm. This is a dynamic test, conducted over 5 minutes: the difference in absorption between the start and the end of the test reflecting the production of NADPH. Correction factors are applied for ambient temperature, and the result must be related to the haemoglobin or red cell content of the specimen. It was not feasible to measure either variable in the field, so haemoglobin was estimated from the haematocrit (spun PCV) of the study subjects, using data from a separate survey in which a full blood count was performed on all subjects. Regression of haemoglobin on calculated haematocrit in that data set yielded Hb(g/dl)=0.03059506x Hct-0.03775. Where the haematocrit for an individual was missing, a haemoglobin value calculated from the mean haematocrit (39.5) of the sample of subjects assayed for G6PD activity was used. There were a number of issues with this method in the field, mostly revolving around voltage stability and differences in pre and post sample blank readings. These are discussed further in Chapter 4.

Chapter 3 – The First Survey

Introduction

Bình Phước province had been selected as the study site on the basis of locally available population statistics and malaria prevalence data. In order to design the planned case-control and cohort studies additional information was required in four key areas. More extensive malaria prevalence data with finer granularity in geospatial and demographic variables was essential to confirm and quantify the local perceptions of a negative correlation between smear positive prevalence and distance from the Cambodian border, and a higher burden of malaria amongst ethnic minority groups. Larger surveys of haemoglobinopathy prevalence were also required, including collection of sufficient volume of blood to allow detection of common alpha thalassaemia mutations by PCR. Achieving a robust case definition of malaria demands a locally relevant understanding of the relationship between parasitaemia and symptoms, especially in children, and collection of good clinical data during malariometric surveys can assist in developing such an understanding. Finally we needed information on health care seeking behaviour, with particular emphasis on the perinatal period. A large cross sectional survey was conducted in February 2000 in three communes in three different districts of Bình Phước province to fulfil these requirements.

Survey methods have been detailed in chapter 2. The survey was actually conducted by teams recruited from HTD, IMPE-HCMC and local health services. Dr Hiền and Dr Phú were extensively involved in both the organisation and practical aspects of this first survey. Other teams members were drawn from the list of those acknowledged in the declaration. The blood smears were read by Miss Ly, Miss Kim and Miss Điệp. The HPLC analysis and α thalassaemia genotyping were carried out by Katie Miles in Oxford. My role in this survey was broad design of the survey goals, including stipulation of the survey sample sizes and specimens to take, design of the survey form, supervision of the survey conduct

to the extent possible (see methods – specific problems for details), and analysis of the data, which was entered by myself and Bùi Văn Minh.

Results

Demographics

A full understanding of the sample demographics is essential to the interpretation of differences in malaria prevalence and the analysis of potential confounders, so they are presented here in some detail. A total of 4184 individuals took part in the survey. The planned sample size totalled 2550. The excess was concentrated in the 15-40 age groups (table 3.1), and Đắc Ơ contributed 39% of the sample compared to 30% from Đồng Tâm and 31% from Đak Nhau. The ethnic composition of the sample is shown in table 3.2. Those ethnic groups which comprise less than 2% of the sample have been grouped as "other". A complete breakdown by hamlet and ethnic group is provided in table 2, appendix 2. Although demographic data from the 1999 census are readily available at provincial level, extracting population breakdown by age, sex or especially ethnic group at the district or commune level has proved extremely difficult. The proportions of different ethnic groups comprising the commune populations was eventually procured for Phước Long district, but not for Đồng Phú or Bù Đăng. It has not been possible to obtain population breakdown by age and ethnicity. A comparison between the age and sex distribution of Bình Phước province and our sample is shown in fig 3.1. There appears to be a relative dearth of adult men of working age, and the intentional excess of children is apparent, but these apart, the eventual sample appears representative of the provincial population in terms of age and sex (although deviating from the planned sampling strategy).

Age Group	Village			Total
	Đắc Ơ	Đồng Tâm	Đak Nhau	
<1	55	56	56	167
1	70	55	64	189
2	75	54	59	188
3	80	53	61	194
4	79	52	57	188
5	81	82	55	218
6	72	55	62	189
7	44	58	49	151
8	71	49	51	171
9	47	36	47	130
10-14	163	131	135	429
15-19	93	44	62	199
20-29	204	162	167	533
30-39	179	144	115	438
40-49	121	107	81	309
50-59	94	61	70	225
60-69	58	46	58	162
70+	36	29	39	104
Total	1,622	1,274	1,288	4,184

Table 3.1: Composition of sample by sampling age group

	Đắc Ơ	Đồng Tâm	Đak Nhau	Total
Kinh	691 (42.6%)	518 (40.7%)	436 (33.9%)	1645 (39.3%)
S'tiếng	784 (48.3%)	254 (19.9%)	91 (7.1%)	1129 (27.0%)
Tày	68 (4.2%)	268 (21.0%)	87 (6.8%)	423 (10.1%)
Nùng	46 (2.8%)	181 (14.2%)	43 (3.3%)	270 (6.5%)
M'Nông	0	1 (0.1%)	581 (45.1%)	582 (13.9%)
Other	33 (2.0%)	52 (4.1%)	50 (3.9%)	135 (3.2%)
Total	1622 (38.8%)	1274 (30.5%)	1288 (30.8%)	4184

Table 3.2: Ethnic composition of sample. Cell contents are number (percentage of commune sample), or number (percentage of total sample) for total rows.

	Sample	Population
Kinh	691 (42.7%)	4416 (52.0%)
S'tiếng	781 (48.2%)	3628 (42.8%)
Tày	68 (4.2%)	577 (6.8%)
Nùng	46 (2.8%)	
Others	33 (2.0%)	71 (0.8%)

Table 3.3: Ethnic breakdown of Đắc Ơ population and February 2000 survey sample from Đắc Ơ.

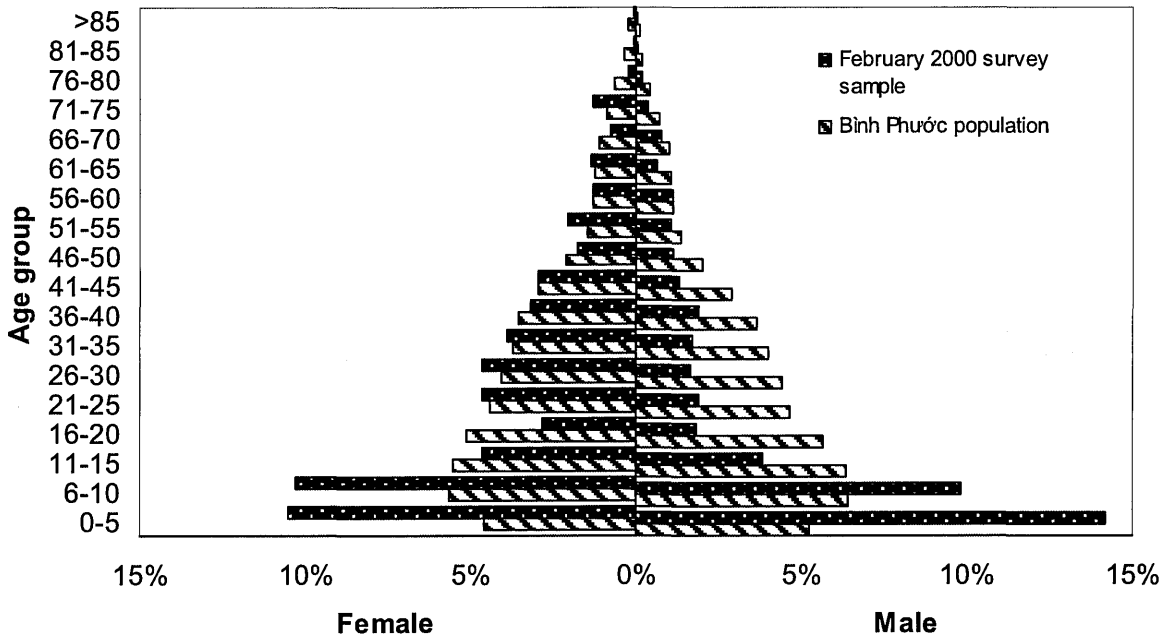


Fig 3.1: Comparison of the survey sample with an expected sample drawn from the population of Bình Phước province.

Đắc Ơ is the only commune in this survey for which the ethnic composition is available, and table 3.3 shows the difference between the population and sample ethnic breakdowns. Minority groups, particularly the S'tiếng, are over-represented in our sample (discussed below). There are small differences in the age and sex distribution between the ethnic groups, with a greater proportion of infants and older adults amongst the ethnic minority groups (table 3.4 and figs 3.2 & 3.3). These differences are smaller than might have been predicted on the basis of national age and ethnic group data and assumptions about reproductive behaviour. The net effect on the mean age in each ethnic group is shown in table 3.5 with the mean age of the national population. The inter-ethnic differences appear to run contrary to national trends. Figure 3.4 depicts the deviation of the sample from the percentage of the national population of each ethnic group in that age range, and demonstrates the relative excess of Kinh children and ethnic minority aged responsible for the apparent sampling error.

	Kinh	S'tiếng	Tày	Nùng	M'Nông	Other	Total
<1	43 (2.6%)	53 (4.7%)	11 (2.6%)	14 (5.2%)	39 (6.8%)	3 (2.2%)	163 (3.9%)
1	71 (4.4%)	52 (4.7%)	22 (5.2%)	12 (4.5%)	27 (4.7%)	6 (4.5%)	190 (4.6%)
2-4	221 (13.5%)	166 (14.9%)	40 (9.5%)	32 (11.9%)	88 (15.4%)	19 (14.2%)	566 (13.6%)
5-9	357 (21.9%)	201 (18.0%)	94 (22.3%)	56 (20.8%)	107 (18.7%)	35 (26.1%)	850 (20.5%)
10-14	203 (12.4%)	110 (9.9%)	38 (9.0%)	20 (7.4%)	40 (7.0%)	17 (12.7%)	428 (10.3%)
15-19	83 (5.1%)	54 (4.8%)	13 (3.1%)	13 (4.8%)	31 (5.4%)	4 (3.0%)	198 (4.8%)
20-29	199 (12.2%)	150 (13.4%)	60 (14.3%)	35 (13.0%)	69 (12.0%)	15 (11.2%)	528 (12.7%)
30-39	186 (11.4%)	99 (8.9%)	53 (12.6%)	35 (13.0%)	48 (8.4%)	11 (8.2%)	432 (10.4%)
40-49	135 (8.3%)	77 (6.9%)	28 (6.7%)	18 (6.7%)	38 (6.6%)	8 (6.0%)	304 (7.3%)
50-59	63 (3.9%)	64 (5.7%)	30 (7.1%)	18 (6.7%)	39 (6.8%)	11 (8.2%)	225 (5.4%)
60-69	49 (3.0%)	49 (4.4%)	15 (3.6%)	14 (5.2%)	28 (4.9%)	4 (3.0%)	159 (3.8%)
70+	22 (1.4%)	42 (3.8%)	17 (4.0%)	2 (0.7%)	19 (3.3%)	1 (0.8%)	103 (2.5%)
Total	1632	1117	421	269	573	134	4146

Table 3.4: Age composition of ethnic group samples: number (percentage of ethnic subsample).

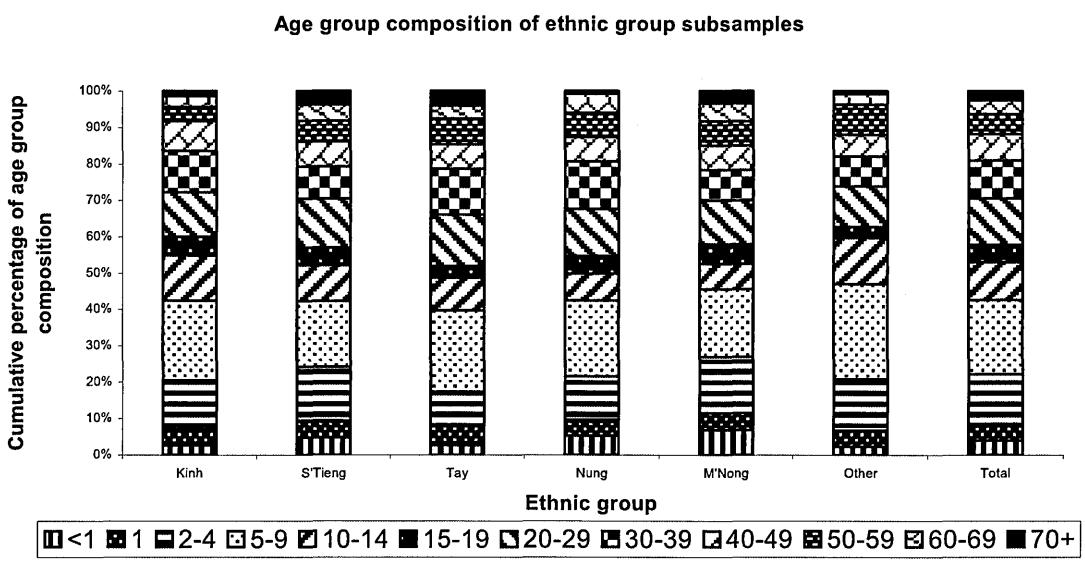


Fig 3.2: Age composition of ethnic group subsamples

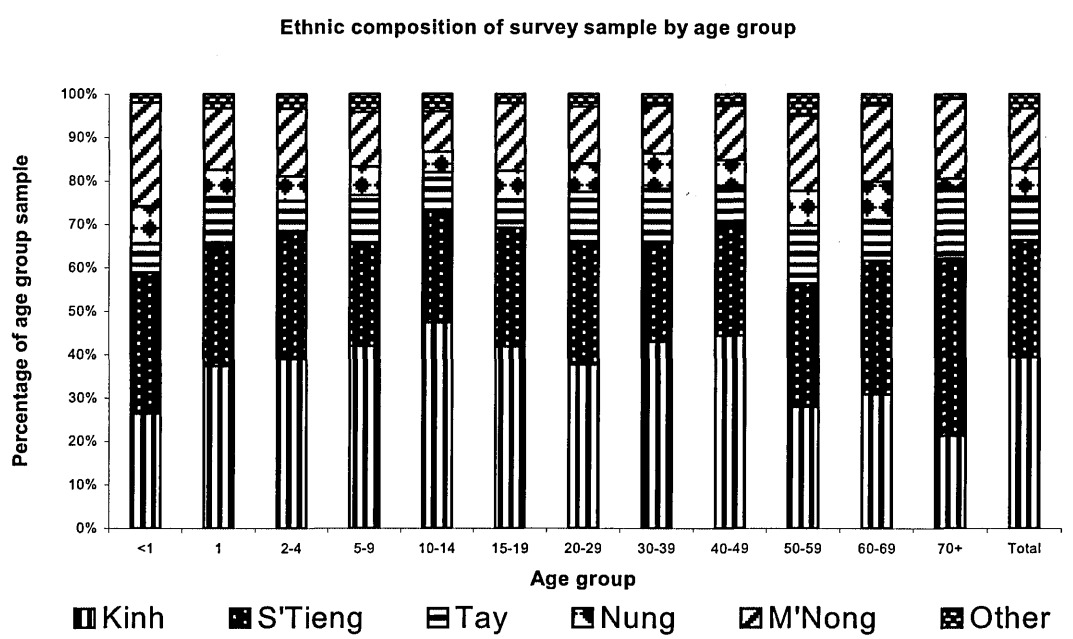


Fig 3.3: Ethnic group contribution to age group subsamples

	Mean age of sample	Mean age of population	p
Kinh	19.7	27.4	<0.0001
S'tiếng	21.4	22.9	0.01
Tày	23.4	25.6	0.03
Nùng	21.3	24.8	0.004
M'Nông	21.1	22.5	0.10
Other	19.2	N/A	
Total	20.8	N/A	

Table 3.5: Comparison between mean age of sample and mean age of national population by ethnic group. p value for T test of national mean=mean of sample.

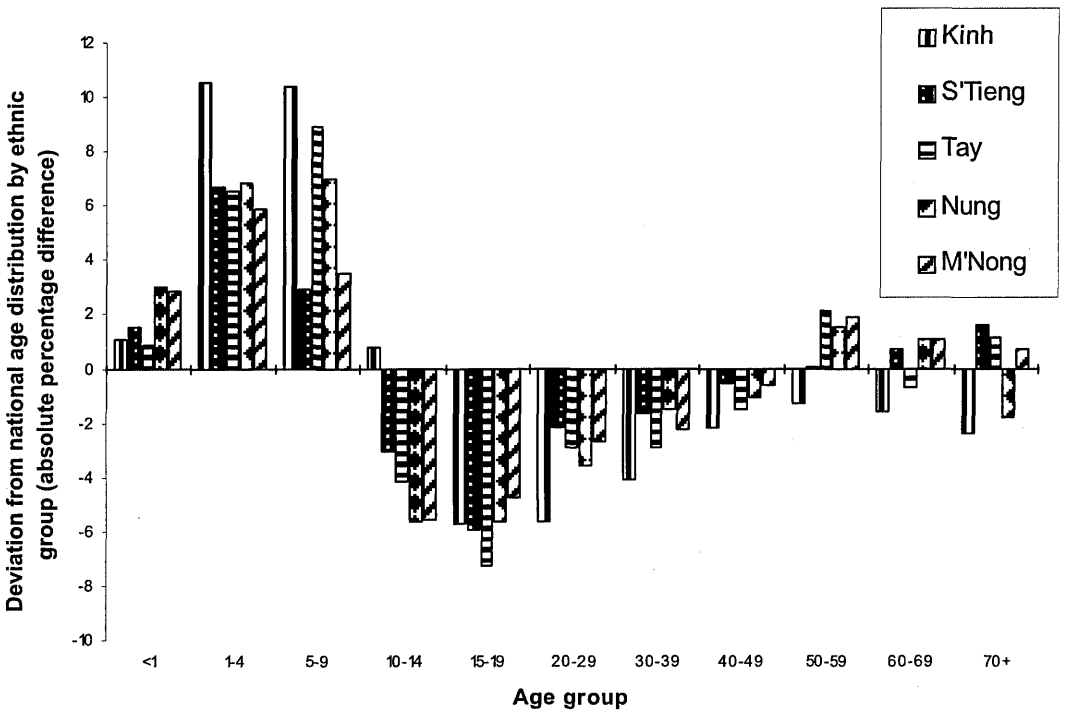


Fig 3.4: Comparison between age distributions of sample and national population, by ethnic group.

Hamlet Name	First survey subject number	Hamlet population	Proportion of hamlet pop S'tiếng	Proportion of hamlet population participating
Thôn 3	123	962	70%	13%
Thôn 4	284	739	72%	38%
Thôn 6	111	993	72%	11%
Thôn 7	32	507	10%	6%
Thôn 9	152	1436	2%	11%
Bù Bưng	219	643	90%	34%
Bù Cà	191	231	94%	83%
Bù Khơn	116	592	95%	20%
Bù Xia	130	632	0%	21%
Đak Lim	67	1130	0%	6%
Đak U	186	550	8%	34%
Total	1611	8415	43%	19%

Table 3.6. Comparison of population & sample distribution between hamlets in Đắc Ơ.

Sufficient population data to examine the geographical breakdown of the sample was only available for Đắc Ơ. Table 3.6 shows the uneven distribution of the sample between the 11 hamlets in Đắc Ơ. Our first impressions were of greater participation in the S'tiếng hamlets, reflected in the increased proportion of S'tiếng individuals in the survey (0.48 compared with 0.43 in the commune population), but there is no significant correlation between the proportion of the hamlet population included in the sample and the proportion of S'tiếng in that hamlet (ρ=0.50, p=0.125).

Blood smear results

A total of 570 individuals had a positive blood smear. Light microscopy speciation attributed 60% of these infections to *P. falciparum* (*Pf*) alone, with 24% due to *P. vivax* (*Pv*) alone, and the remainder predominantly mixed vivax and falciparum. Thirteen individuals were harbouring *P. malariae* parasites, seven of these appearing as single infections. Two cases of *P. ovale* infection were identified, both with concomitant falciparum. *P. ovale* infection is rare in this region, although has been reported from Thailand (Cadigan et al. 1969; Zhou et al. 1998). This was the first time that *P. ovale* had been documented in southern Vietnam, although this was not altogether surprising given the size of the sample and the diligence of the microscopists. The results are summarised in table 3.7. Patterns in species prevalence and gametocyte carriage are discussed below.

	Đắc Ơ	Đồng Tâm	Đak Nhau	Total
Total smear positive	320 (19.7%)	130 (10.2%)	124 (9.6%)	574 (13.7%)
Falciparum	162 (50.6%)	68 (52.3%)	66 (53.2%)	296 (51.6%)
Falciparum gametocyte only	28 (8.8%)	9 (6.9%)	11 (8.9%)	48 (8.4%)
Vivax	71 (22.2%)	30 (23.1%)	37 (29.8%)	138 (24.0%)
Malariae	3 (0.9%)	4 (3.1%)	0	7 (1.2%)
Falciparum & Vivax	48 (15.0%)	19 (14.6%)	10 (8.1%)	77 (13.4%)
Falciparum & Malariae	5 (1.6%)	0	0	5 (0.9%)
Falciparum & Ovale	2 (0.6%)	0	0	2 (0.3%)
Vivax & Malariae	1 (0.3%)	0	0	1 (0.2%)

Table 3.7. Summary of smear results by commune. Percentages are of commune sample for total smear positive, and of total smear positive for species. No individuals harboured vivax or malariae gametocytes alone.

Geography and ethnicity

There was considerable heterogeneity in smear positive prevalence between ethnic groups, hamlets and age groups, with a small sex difference. The S'tiếng suffered the highest prevalence of malaria (26%), followed by the M'Nông (12%). An unexpected finding was the higher prevalence of parasitaemia in the Kinh (10%), than in the Tày (3.5%) and Nùng (6%), the other two ethnic minority groups present in any number in the sample. The ethnic minority groups were unevenly distributed between the hamlets (Table 3.10), raising geographical variation in transmission as a possible confounder. The smear positive prevalence did differ between communes, that in Đắc Ơ being double those of Đồng Tâm and Đak Nhau (table 3.7). There was even greater variation between hamlets (fig 3.5), even within communes, at which level it becomes difficult to disentangle the effects of ethnicity from ecological microvariations in transmission, as few hamlets are of mixed ethnicity.

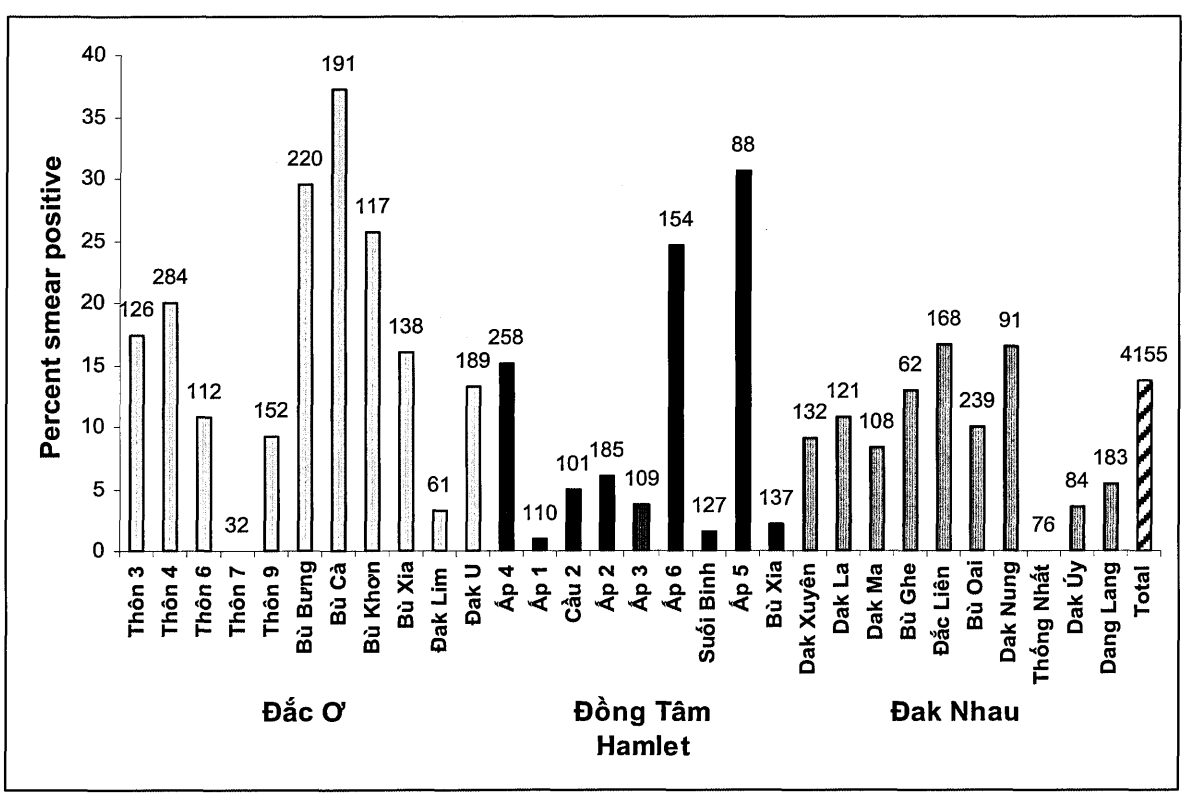


Fig 3.5. Percent smear positive by hamlet. Bar labels indicate total number of observations in that hamlet.

Examining the malaria prevalence across hamlets, accounting for ethnic group, two trends are apparent: there is greater malaria transmission in Đắc Ơ than in the other communes, and there do appear to be high and low risk hamlets. Analysing only Kinh subjects, to avoid any ethnic group effects, Dak Lim in Đắc Ơ has significantly less malaria than the rest of the commune (1/73 vs 72/618 p=0.03), whilst Ấp 4 (24/143 vs 34/375 p=0.02) and Ấp 6 (20/87 vs 38/431 p=0.001) in Đồng Tâm and Dac Lien in Đak Nhau (7/24 vs 25/412, p=0.001) have significantly more (although the numbers in Đak Nhau are small). Although the average ethnic specific SPP for the Kinh in Đắc Ơ is no different to those of Đak Nhau and Đồng Tâm, the even spread of moderate amounts of malaria amongst the Kinh across most of the hamlets in this commune may indicate higher transmission rather than sampling error. The S'tiếng, Tày and Nùng data reinforce this suspicion: subjects from Đắc Ơ belonging to all three of these ethnic groups have significantly more malaria than those from the other two communes (table 3.8).

	Kinh	S'tiếng	Tày	Nùng
Đắc Ơ	73/691 (10.6%)	231/784 (29.5%)	8/68 (11.8%)	6/45 (13.0%)
Other communes	90/955 (9.4%)	68/345 (19.7%)	7/355 (2.0%)	10/225 (4.4%)
p value	0.445	0.001	<0.001	0.024

Table 3.8: Within ethnic group comparison of malaria prevalence between Đắc Ơ and the other 2 communes. Cell contents are number smear positive/total number of observations in commune ethnic subsample (percent smear positive). M'Nông are not shown as they only reside in Đak Nhau.

Age

The relationship between SPP and age is depicted in fig 3.6. The relative protection of infants and young children, the rise through childhood and into early adulthood, followed by decline and plateau through to old age is clearly apparent. Examining this relationship in the different ethnic groups shows a rather different pattern, however (fig 3.7). The Kinh show a much flatter curve, with a peak in the 15-19 age group and a plateau near the peak value. The peak in the S'tiếng is earlier (in the 5-9 year olds), much more pronounced, and with a steeper decline to a plateau well below the peak values, although above level of the

Kinh plateau. The M'Nông show an intermediate pattern, with a peak at 5-9, and greater shape to the curve than the Kinh but less than the S'tiếng. The number of smear positive individuals in the other groups is too small to allow any patterns to be discerned reliably. S'tiếng smear positive subjects were younger (mean age 14.7 years, p=0.0003 vs all other ethnic groups, p=0.0002 vs Kinh), as were M'Nông (14.2, p vs all=0.12, p vs Kinh=0.002), whilst Tày (28.4, p vs all=0.006, p vs Kinh 0.08) and Nùng (26.8, p vs all=0.01, p vs Kinh=0.14). The mean age of smear positive Kinh subjects was 20.6 years.

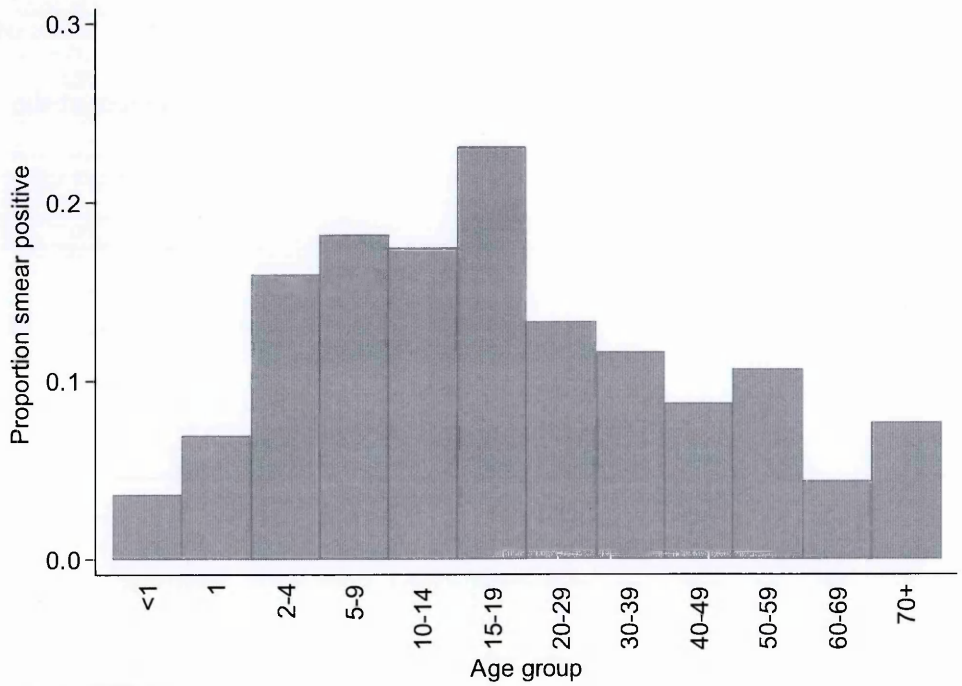


Fig 3.6. SPP by age

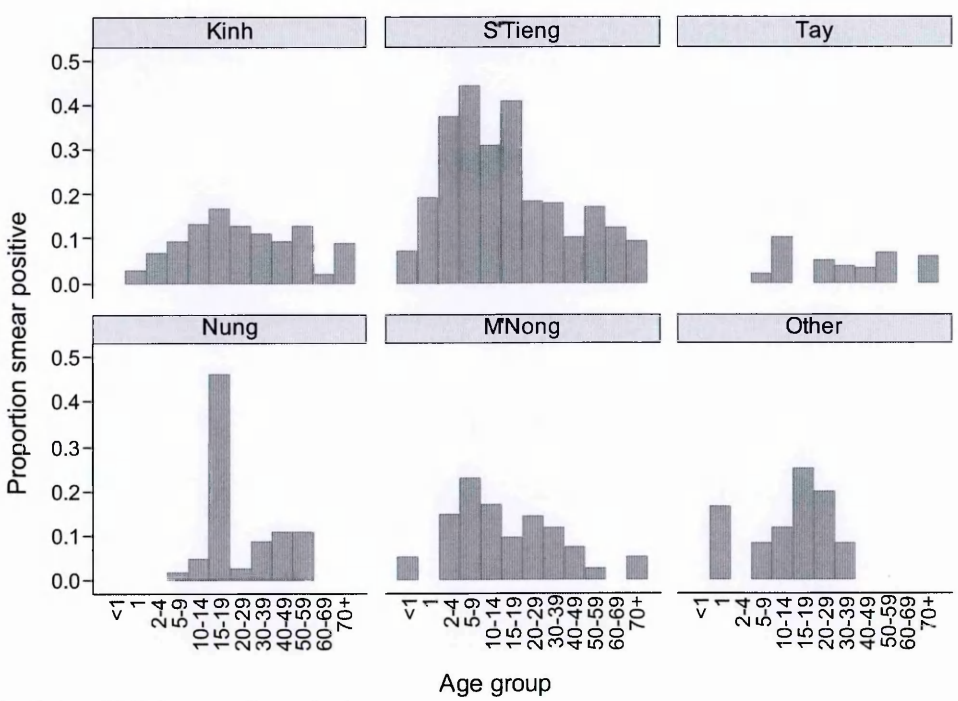


Fig 3.7. SPP by age by ethnic group

Sex

Men were at greater risk than women for harbouring parasites (15.0% vs 12.5% p=0.02). This effect was not apparent in all ethnic groups: there was no significant effect in the Kinh or the Tày, an effect of borderline significance in the S'tiếng, and a significant effect in the Nùng and M'Nông (table 3.9). Once again geography and ethnicity confound one another. There was no effect apparent in Đắc Ơ, with moderate effects in Đồng Tâm and Đak Nhau (table 3.9). The ethnic groups showing the largest sex difference are concentrated in particular communes, thus it was not possible to gain an insight into the relative role of geography and ethnicity by examining patterns within ethnic groups across communes. The sex differential was also inconsistent across age groups (table 3.10). There was no sex difference in children (a trend towards a higher risk in girls was apparent in some age groups). The difference becomes apparent in the 15-19 year olds, and remains a feature through the remainder of life. Examining Kinh and S'tiếng separately, there is a trend, occasionally reaching significance, towards a higher prevalence in men in most of the adult age groups, but this is offset by a significantly higher risk for school age girls. As the bulk of infections occurs in this age group, the net effect is no apparent sex differential. Fig 3.10 depicts sex ratios in SPP across age groups for Kinh, S'tiếng, Nùng and M'Nông.

Ethnic group	SPP		p
	Male	Female	
Kinh	10.1%	9.8%	0.83
S'tiếng	28.7%	24.0%	0.08
Tày	3.4%	3.7%	0.85
Nùng	9.6%	3.2%	0.03
M'Nông	16.2%	9.3%	0.01
Other	9.3%	7.4%	0.70
Total	15.0%	12.5%	0.02
Village			
Đắc Ơ	20.1%	19.6%	0.80
Đồng Tâm	11.5%	8.0%	0.04
Đak Nhau	12.3%	7.7%	0.005

Table 3.9. Smear positive prevalence by sex in the different ethnic groups and villages.

Age group	SPP		p
	Male	Female	
<1	1.2%	5.0%	0.15
1	7.6%	4.8%	0.44
2-4	15.8%	15.3%	0.86
5-9	15.7%	20.1%	0.10
10-14	18.5%	15.9%	0.49
15-19	32.5%	16.8%	0.01
20-29	18.6%	11.3%	0.03
30-39	13.8%	10.6%	0.32
40-49	12.4%	6.6%	0.08
50-59	15.5%	7.8%	0.07
60-69	6.9%	2.3%	0.15
70+	5.4%	9.0%	0.52

Table 3.10. Effect of age on sex differential in smear positivity

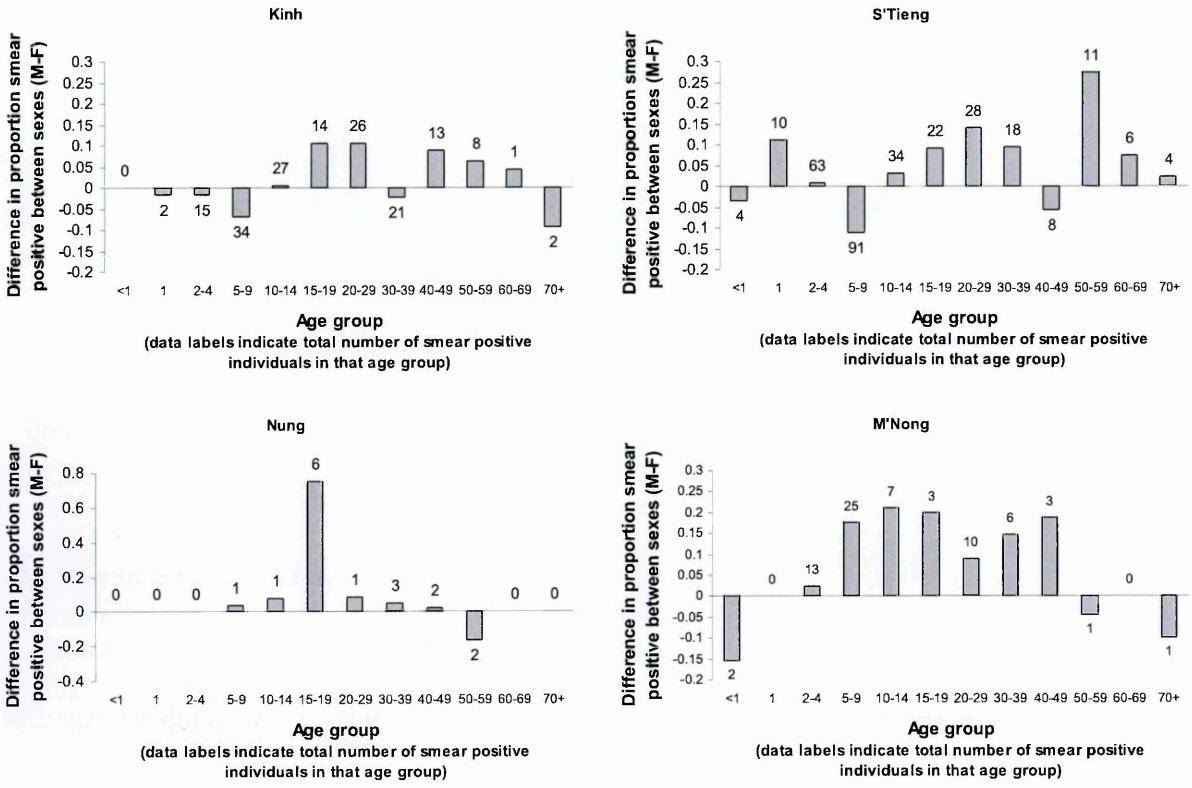


Fig 3.8. Variation in sex difference in smear positivity with age and ethnicity

Malaria and pregnancy

Fifty nine pregnant women were included in the survey, although only 274 of the 953 women of reproductive age had a definitive pregnancy status recorded. Pregnant women were not at significantly higher risk for malaria in comparison with all survey subjects, but were at significantly higher risk when compared to women of reproductive age, and the odds ratio in comparison with non-pregnant women of reproductive age was amongst the highest of any variable examined in this survey (table 3.11). Only the prevalence of *P. falciparum* was increased in pregnant women: the prevalences of *P. vivax* and mixed infections were the same.

Comparator group	Odds ratio	p value
All	1.4	0.27
Women of reproductive age	2.0	0.05
Non-pregnant women of reproductive age	3.4	0.003

Table 3.11. Risk of parasitaemia in pregnant women vs specified comparator group.

Species

Vivax infection was generally more frequent in the younger age groups: in particular the 5-9 year olds had significantly more vivax than other ages (table 3.12). This age related difference was less apparent when infections with vivax alone were considered, although this group is still statistically significantly younger than those with other types of infection (mean age 14.1 +/-0.98 SE vs 18.7 +/- 0.89 p=0.001). A corollary (or possibly the cause) of this relationship is the higher proportion of mixed infections in children and young adults. There was no relationship between sex and species. There were some ethnic perturbations in species distribution, however, some of which reached statistical significance (table 3.13). Almost all the smear positive Nùng were infected with vivax, which did not initially appear to be a geographical cluster, as the 14 smear positive Nùng came from all 3 communes. Five of those with pure vivax infection appear to come from two families, however, reducing the number of independent observations. As one of these families also contributed a pure falciparum infection, and a third family contributed both a pure falciparum and a mixed falciparum/vivax infection, this anomaly cannot be taken as a robust result as it is based on only 3 families. The predilection for vivax remains statistically significant even if all observations from these families are excluded, however, albeit now based on only 8 smear positive individuals, so merits examination in subsequent data. The small difference in the proportion of pure vivax infections between the S'tiếng and other ethnic groups reached statistical significance, although the difference in all vivax infections did not. There were fewer mixed infections in the M'Nông, and fewer than expected in Đak Nhau. Once again it is difficult to disentangle the effects of geography and ethnicity, as almost all the M'Nông live in Đak Nhau. Although not statistically significant, the Kinh in Đak Nhau had fewer mixed infections than the Kinh in other hamlets (3/436 vs 24/1209 observations, p=0.08, 3/32 vs 24/131 smear positive, p=0.29), but this was not true of the S'tiếng or other ethnic groups, suggesting that geography might play a role. The proportions of mixed infections amongst non-M'Nông are consistent

across hamlets in Đak Nhau, which does not suggest any microepidemiological effect, and there did not appear to be any separation of M'Nông vivax and falciparum infections into different hamlets (appendix 2 table 3). The M'Nông smear positive subjects were younger than Kinh, Tày and Nùng, but the same age as the S'tiếng (summary of means appendix 2 table 4, full breakdown table 5). The level of parasitaemia was also comparable.

Age Group	Smear positive	Falciparum alone	Vivax alone	Mixed infections	Gameto-cytaemia	Any falciparum	Any vivax
<1	6 (3.6%)	3 (50%)	3 (50%)	0	1 (17%)	3 (50%)	3 (50%)
1	13 (6.9%)	8 (62%)	4 (31%)	1 (8%)	8 (61%)	9 (69%)	5 (39%)
2-4	91 (16.0%)	41 (45%)	30 (33%)	20 (22%)	38 (42%)	61 (67%)	47 (52%)
5-9	156 (18.2%)	92 (59%)	36 (23%)	26 (17%)	53 (34%)	118 (76%)	59 (38%)
10-14	75 (17.5%)	47 (63%)	15 (20%)	11 (15%)	28 (37%)	58 (77%)	26 (35%)
15-19	46 (23.1%)	22 (48%)	14 (30%)	9 (20%)	17 (37%)	31 (67%)	23 (50%)
20-29	71 (13.3%)	46 (65%)	13 (18%)	10 (14%)	26 (37%)	55 (77%)	23 (32%)
30-39	51 (11.6%)	39 (77%)	8 (16%)	3 (6%)	15 (29%)	42 (82%)	10 (20%)
40-49	27 (8.7%)	18 (67%)	8 (30%)	1 (4%)	8 (30%)	19 (70%)	9 (33%)
50-59	24 (10.7%)	17 (71%)	5 (21%)	2 (8%)	5 (21%)	19 (79%)	7 (29%)
60-69	7 (4.3%)	6 (86%)	1 (14%)	0	2 (29%)	6 (86%)	1 (14%)
70+	8 (7.7%)	5 (62%)	1 (13%)	1 (13%)	2 (25%)	6 (75%)	2 (25%)
Total	575 (13.7%)	344 (59.8%)	138 (24.0%)	84 (14.6%)	203 (35.3%)	427 (74.3%)	215 (37.4%)

Table 3.12. Age group and some species indicators. Cell contents are number (percent) positive. Percentages in the "Smear positive" column refer to a denominator of all individuals in that age group. Percentages in the other columns refer to a denominator of smear positive individuals in that age group. The 7 isolated *P. malariae* infections are not shown as they are too few to examine any age variation. Mixed *P. malariae* and *P. ovale* infections are included in mixed infections.

EG	Smear positive	Falciparum alone	Vivax alone	Mixed infections	Gameto-cytaemia	Any falciparum	Any vivax
Kinh	163 (9.9%)	97 (59%)	37 (23%)	27 (17%)	56 (34%)	124 (76%)	64 (39%)
S'tiếng	299 (26.5%)	187 (63%)	60 (20%)	49 (16%)	114 (38%)	235 (79%)	102 (34%)
Tày	15 (3.5%)	7 (47%)	4 (27%)	3 (20%)	6 (40%)	10 (67%)	7 (47%)
Nùng	16 (5.9%)	4 (25%)	10 (63%)	2 (13%)	5 (31%)	6 (38%)	12 (75%)
M'Nông	71 (12.2%)	42 (59%)	26 (37%)	3 (4%)	19 (27%)	45 (63%)	29 (41%)
Other	11 (8.1%)	7 (64%)	1 (9%)	0	3 (27%)	7 (64%)	1 (9%)
Total	575 (13.7%)	344 (59.8%)	138 (24.0%)	84 (14.6%)	203 (35.3%)	427 (74.3%)	215 (37.4%)

Table 3.13. Ethnic group and some species indicators. Cell contents are number (percent) positive. Percentages in the "Smear positive" column refer to a denominator of all individuals in that ethnic group. Percentages in the other columns refer to a denominator of smear positive individuals in that ethnic group. The 7 isolated *P. malariae* infections are not shown as they are too few to examine any ethnic variation. Mixed *P. malariae* or *P. ovale* infections are included in mixed infections.

Parasitaemia

Parasitaemia was only assessed for pure falciparum infections. Parasite counts per 400 white blood cells or 1000 red blood cells were converted to parasites per microlitre by assuming an average WBC count of $8x10^9$/l and an average RBC count of $4x10^{12}$/l. Parasite counts were then log transformed for further analysis. The S'tiếng and M'Nông tended to have lower parasite counts than the other ethnic groups (fig 3.9), but none of these differences reached significance. There was a relationship between age and parasite count (fig 3.10). Thirty to thirty nine year olds and the over 70's had significantly lower parasitaemias, and the overall trend to decreasing parasitaemia with increasing age was significant (linear regression coefficient –1.23, p=0.003). The relationship was less strong in the Kinh than the S'tiếng and M'Nông (fig1 appendix 2), and linear regression of parasitaemia on age remained significant only for the S'tiếng.

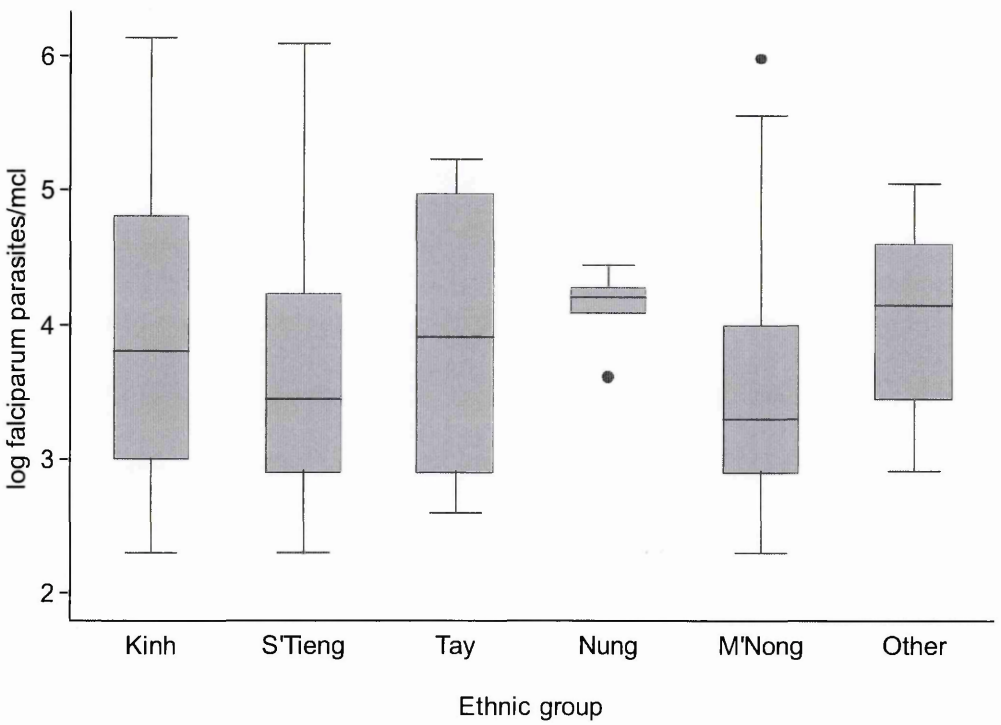


Fig 3.9. Log parasitaemia by ethnic group

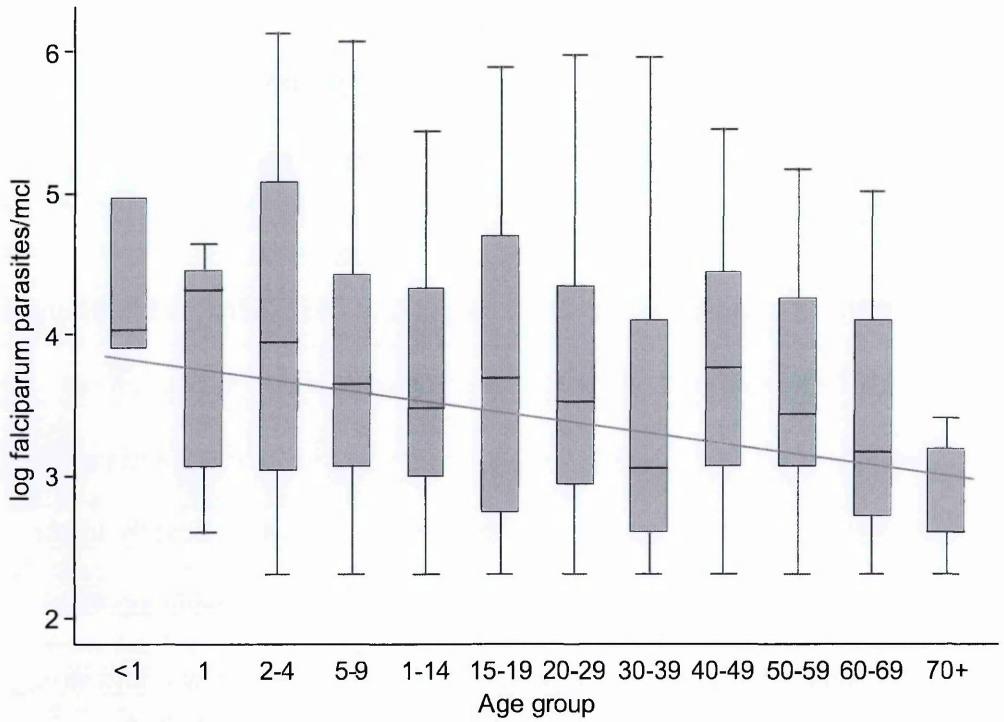


Fig 3.10. Log parasitaemia by age group (linear regression coefficient –1.23, p=0.003).

Gametocyte carriage

Approximately one third of smear positive individuals demonstrated gametocytes on peripheral smear. The proportion was higher for falciparum than vivax (42% vs 13.5%, $p<0.001$). There were no sex, commune or ethnic groups differences in gametocyte carriage, but there was an effect of age (fig 3.11). The obvious confounder is the higher parasitaemias found in the younger age groups. Both remained significant in multiple logistic regression analysis. Within age groups there were some statistically significant sex differences – a female predilection for gametocytaemia in the 10-14 year olds balanced by a male bias in the 15-19 year olds. There were no ethnic specific age differences, and no significant differences in the relationship between age and gametocyte carriage between the major ethnic groups (fig 2 appendix 2).

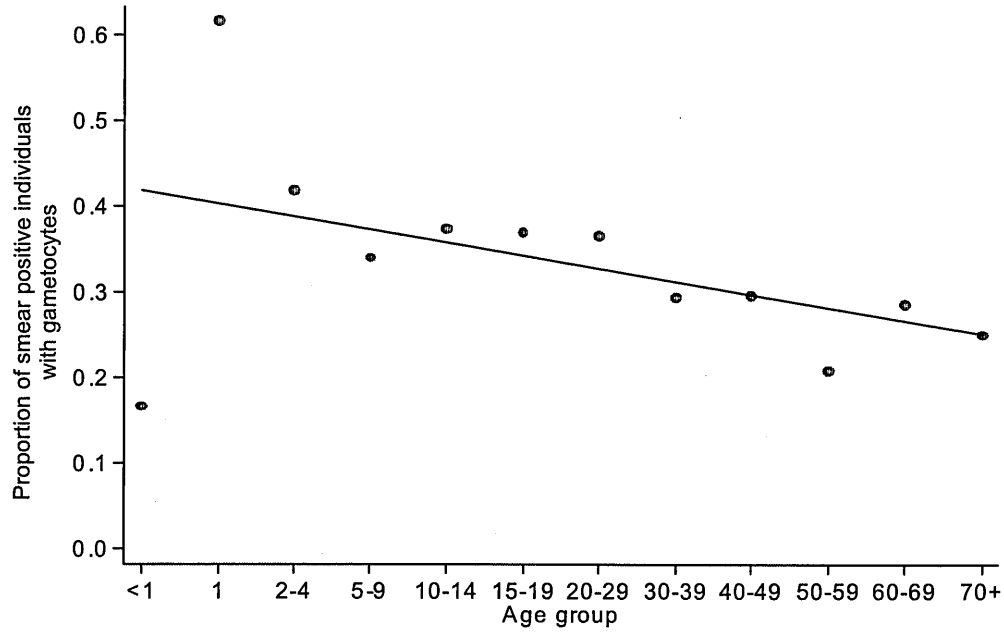


Fig 3.11. Effect of age on gametocyte carriage (linear regression coefficient –0.015 for regression on age group, -0.003 for regression on age, both $p<0.001$)

Symptomatology

The primary aim of gathering clinical data on survey subjects was to attempt to distinguish malarial fevers from non-malarial fevers with coincidental parasitaemia. All study subjects were asked whether they had suffered a fever in the preceding 7 days and the preceding 2 days, had their temperatures measured, and were examined for the presence of splenomegaly. Pyrexial individuals, and those reporting fever, were further questioned on the presence of a set of symptoms and examined for specific signs. These symptoms and signs were selected either to provide support to a diagnosis of malarial fever (chills, rigors, headache, pallor, jaundice – "positive features"), or help refute this diagnosis (diarrhoea, upper respiratory tract symptoms, characteristic rash, pharyngitis, inflamed tympanic membrane, chest signs – "negative features"). Only 505 of the 964 subjects reporting fever or found to be febrile at the time of the survey had any symptomatology questions completed, and only 102 had all questions completed, though a further 146 sheets were missing only the respiratory rate. The symptomatology section was also completed for 36 afebrile individuals not reporting fever. Results from these individuals are not presented below unless specifically noted.

The most frequently reported symptoms were headache, which was almost universal amongst adult subjects with symptom data, and coryza. Whilst this might have been expected, the high incidence of rigors (44%) and chills (52%) was not. Both these symptoms were also more evenly distributed than anticipated across age groups (full details of age group and symptomatology are given in table 6 appendix 2). As many adults complained of sore throat as children, although ear localising symptoms were more frequent in 2-9 year olds. The presence of rash was noted in only 2 individuals in the whole survey, so no age breakdown is presented. There were no significant sex differences in the incidence of any symptoms. There were unexpected variations in the reported incidences of different symptoms between villages (full breakdown of symptoms and signs by village is presented in table 8, appendix 2), which are discussed below.

Only a third of the smear positive individuals reported fever or were pyrexial at the time of the survey. Although reported and measured fever, splenomegaly, rigors, chills, headache and pallor were all significantly associated with parasitaemia, and coryza, and the presence of pharyngitis on examination significantly negatively associated with parasitaemia (tables 9 and 10, appendix 2), robustly distinguishing malaria fevers from non-malaria fevers proved impossible with the clinical data collected. The prevalence of rigors, chills and headache was so high amongst both smear positive and negative individuals, that the specificity and sensitivity of any of these features as indicators that a fever was due to malaria were very low (tables 3.14 and 3.15).

Clinical feature	Sensitivity	Specificity	PPV	NPV
Diarrhoea	0.88	0.15	0.27	0.78
>5 stools	0.96	0.03	0.25	0.71
Sore throat	0.75	0.29	0.28	0.76
Earache/discharge	0.97	0.03	0.27	0.75
Coryza	0.56	0.56	0.32	0.78
Pharyngitis	0.82	0.02	0.27	0.80
Inflamed TM	0.80	0.21	0.28	0.79
Chest signs	0.98	0.06	0.27	0.89

Table 3.15. Sensitivity, specificity, positive and negative predictive values for each negative symptom as a "test" for the absence of parasitaemia.

Clinical feature	Sensitivity	Specificity	PPV	NPV
Fever in past 2 days	0.26	0.88	0.26	0.88
Fever in past 7 days	0.19	0.88	0.19	0.87
Reported fever	0.32	0.82	0.22	0.88
Measured fever	0.18	0.94	0.31	0.88
Fever (measured or reported)	0.36	0.79	0.22	0.89
Splenomegaly	0.35	0.93	0.46	0.90
Rigors	0.61	0.63	0.37	0.82
Chills	0.71	0.55	0.35	0.84
Jaundice	0.09	0.90	0.24	0.73
Pallor	0.32	0.87	0.47	0.77
Diarrhoea	0.12	0.85	0.22	0.73
>5 stools	0.04	0.97	0.29	0.75
Sore throat	0.25	0.71	0.24	0.72
Earache or aural discharge	0.03	0.97	0.25	0.73
Coryza	0.44	0.44	0.22	0.68
Pharyngitis	0.02	0.78	0.20	0.73
Inflamed TM	0.15	0.74	0.21	0.72
Abnormal chest auscultation	0.02	0.94	0.11	0.73

Table 3.14. Sensitivity, specificity, positive and negative predictive values for each symptom as a "test" for malaria. "Negative" symptoms are below the bar, positive above.

Haemoglobinopathies

A total of 3928 individuals had their haemoglobin successfully characterised by dedicated HPLC analysis. The investigators in Vietnam were blind to individual's haemoglobinopathy status, in order to prevent any possibility of influencing the course of the case control and cohort studies should any survey subject reappear in a subsequent project, but did receive aggregate results by ethnic group. These results are shown in table 3.16. HbE is in Hardy-Weinberg equilibrium in all ethnic groups with more than ten observations (observed numbers match expected numbers exactly), although obviously not in the population as a whole. No HbC, HbD or HbS was found. A number of samples displayed some minor peaks, which might represent variant haemoglobins, or, more probably, degradation products from suboptimal specimen handling. Minor variant haemoglobins were of no interest to this programme of study, so these peaks were not worked up further. The existence of these peaks in no way affected the assignment of HbE

genotype, or isolated β thalassaemia trait. It became clear that some of the degradation products eluted in the HbF window, however, complicating the establishment of β thal/HbE compound heterozygote genotypes. This was a greater problem in later surveys, and is discussed in more detail in Chapter 4. Genetic analysis for alpha thalassaemias was also performed, but those results were not available for several months, so will be presented in chapter 4. The striking finding from the HPLC analysis was the dramatic difference in prevalence of HbE. The M'Nông, and especially the S'tiếng, have a high carrier frequency of HbE, and very little beta thalassaemia. The Tày, Nùng and Kinh had very little HbE, and more beta thalassaemia. Possible reasons for these differences are explored in chapter 4. The significance of these results here is the impact they had on the design and conduct of further studies.

Ethnic group	Normal	β-thalassaemia trait	HbE heterozygote	HbE homozygote	Total
Kinh	1453 (93.6%)	31 (2.0%)	68 (4.4%)	0	1552
S'tiếng	382 (36.9%)	2 (0.2%)	480 (46.4%)	170 (16.4%)	1034
Mường	9 (90%)	0	1 (10%)	0	10
Tày	367 (89%)	33 (8%)	11 (3%)	0	411
Nùng	209 (90%)	19 (8%)	3 (1%)	0	231
Khmer	30 (60%)	0	12 (24%)	8 (16%)	50
Châu-Ro	0	0	2 (100%)	0	2
M'Nông	317 (57.4%)	2 (0.4%)	202 (36.6%)	31 (5.6%)	552
Chăm	9 (82%)	2 (18%)	0	0	11
Hoa	16 (100%)	0	0	0	16
Ê Đê	0	0	1 (33%)	2 (67%)	3
Dao	16 (89%)	2 (11%)	0	0	18
Sán Dìu	4 (80%)	1 (20%)	0	0	5
Thái	1 (50%)	0	1 (50%)	0	2
Thuong	0	0	1 (100%)	0	1
Tiu	1 (100%)	0	0	0	1
Chao Mạ	2 (67%)	0	1 (33%)	0	3
Total	2816 (67.5%)	92 (2.2%)	783 (18.8%)	211 (5.1%)	3902

Table 3.16. Variant haemoglobinopathy results for all ethnic groups

Place of birth

The accompanying adult relative of any child under 5 years old attending the survey was questioned as to the child's place of birth. Table 3.17 shows the spread of replies by ethnic group. It is immediately clear that the Kinh are much more likely than the minority ethnic

groups to attend a health care centre for delivery (although 40% still deliver at home). There is also an effect of geography (table 3.18), which is only partly attributable to accessibility: as previously mentioned, the health station in Đồng Phú is further from Đồng Tâm than the provincial hospital, and, being so close to a major institution, will take very few deliveries in any case. Bù Đăng district hospital is more accessible from Đak Nhau than Phước Long hospital is from Đắc Ơ, accounting for the lower proportion of women from the latter commune delivering in hospital. Nevertheless, combining both types of health centre, 35% in Đồng Tâm attend either type of health centre peripartum, compared to 25% in Đak Nhau, and 24% in Đắc Ơ. If we restrict our analysis to the Kinh, to avoid intercommune ethnic bias, the difference is even more stark: 76% in Đồng Tâm, compared to 59 and 50% in Đak Nhau and Đắc Ơ respectively. This difference also extends to the S'tiếng, albeit at much lower levels of overall attendance (table 3.19).

Ethnic group	Total <5 yrs	No reply	Home - TBA	Home - alone	Health station	Hospital
Kinh	421	85	95 (28%)	38 (11%)	87 (26%)	116 (35%)
S'tiếng	337	71	164 (62%)	84 (32%)[§]	4 (1%)	14 (5%)
Tày	93	6	21 (24%)	47 (54%)	3 (3%)[*]	16 (18%)
Nùng	69	3	33 (50%)	24 (36%)	2 (3%)	7 (11%)
M'Nông	188	41	127 (86%)	18 (12%)	0	2 (1%)
Other	36	7	9 (31%)	8 (28%)	6 (21%)	6 (21%)
Total	1144	213	449 (48%)	219 (24%)	102 (11%)	161 (17%)

Table 3.17. Place of birth by ethnic group. Percentages are of those with replies. Small case percentages indicate a non-significant difference between that ethnic group and all others. All other p values<0.01 except [§] p=0.01, [*] p=0.04

Village	Total <5	No reply	Home - TBA	Home - alone	Health station	Hospital
Đồng Tâm	352	27	103 (32%)	108 (33%)	6 (2%)	108 (33%)
Đak Nhau	352	88	177 (67%)	20 (8%)	34 (13%)	33 (13%)
Đắc Ơ	440	100	168 (49%)	91 (27%)	62 (18%)	19 (6%)
Total	1144	213	449 (48%)	219 (24%)	102 (11%)	161 (17%)

Table 3.18. Place of birth by commune. Percentages are of those with replies. Small font percentages indicate a non-significant difference between that village and all others. All other p values<0.01.

Village	Ethnic group	Total <5	No reply	Home - TBA	Home - alone	Health station	Hospital
Đồng Tâm	Kinh	123	16	14 (13%)	12 (11%)	5 (5%)	76 (71%)
Đak Nhau	Kinh	119	27	36 (39%)	2 (2%)	29 (32%)	25 (27%)
Đắc Ơ	Kinh	179	42	45 (33%)	24 (18%)	53 (39%)	15 (11%)
Total Kinh		421	85	95 (28%)	38 (11%)	87 (26%)	116 (35%)
Đồng Tâm	S'tiếng	113	6	47 (44%)	47 (44%)	0	13 (12%)
Đắc Ơ	S'tiếng	206	51	113 (73%)	37 (24%)	4 (3%)	1 (1%)
Total S'tiếng		337	71	164 (62%)	84 (32%)[§]	4 (1%)	14 (5%)

Table 3.19. Variation of place of birth within an ethnic group by commune. Percentages are of those with replies. Small font percentages indicate a non-significant difference between that village and all others within that ethnic group. All other p values<=0.01. S'tiếng and Kinh within each village significantly different for all behaviours except Home-alone in Đắc Ơ (NS): all p values<0.01 except Health station in Đồng Tâm p=0.029.

Discussion

Demographics

The planned sampling strategy envisaged deviating from accurate representation of population structure in two regards: enrichment of the sample with children, and a slight bias towards recruiting ethnic minority groups, in particular S'tiếng. The sample achieved was less skewed towards lower age groups than planned, possibly due either to adults accompanying children being recruited, or to the unfocused invitation methods. There was a higher proportion of S'tiếng in the sample than in the population, but it is unclear whether this occurred by design Ethnic minority groups are, on the whole, poorer than the Kinh, and thus more likely to take time away from their routine tasks to attend for a free medical examination. Many hamlets have more than one distinct population centre, and these centres may be dense or more diffuse. The ethnic minority population centres tend to be denser, thus news of the survey may have been transmitted more effectively in these hamlets. The exception is Đắc Ơ, which has a distinct commune centre, based around the market, which is predominantly populated by Kinh. This hamlet was not surveyed, however, as local sources predicted a poor turn out.

The dearth of adult males is a common finding in cross sectional surveys in rural environments, and is usually attributed to this tranche of the population being engaged in farming activities away from the home during the day, and thus not in a position to be surveyed by any method.

Malaria and ethnicity

The differences in prevalence between hamlets combined with the variation of ethnic composition of hamlet populations cause difficulties in establishing the relative importance of ethnicity and local ecology as risk factors for malaria. The association between SPP and the proportion of S'tiếng in hamlet samples is shown in fig 3.12, and that between the SPP and the proportion of S'tiếng in the hamlet population (once again, only for Đắc Ơ) is depicted in fig 3.13.

Deconfounding these relationships poses problems, as the number of hamlets and the fact that very few have a significantly mixed population renders both stratified and multiple regression analysis impotent. A suggestion that the effect of ethnicity might be at least partly independent of geography is the consistency of the smear positivity ranking across all hamlets (table 3.20). The S'tiếng demonstrated a higher SPP in all hamlets with more than 10 S'tiếng subjects with the exception of Ấp 1 and Ấp 2 in Đồng Tâm, and in Đồng Tâm and Đak Xuyền in Đak Nhau, although there were small numbers of S'tiếng in the latter 2 hamlets. The M'Nông also appeared to have a relatively high SPP. The situation for the Tày and Nùng is less clear, as they appeared to be drawn predominantly from hamlets with a lower overall malaria prevalence, and there are fewer hamlets with sufficient numbers of either Tày or Nùng and another ethnic group to allow comment on their position in a comprehensive malaria risk table or disentangle the effect of hamlet and ethnicity. The Tày did appear to have consistently less malaria than the Nùng, however, despite their close relationship with one another (see introduction).

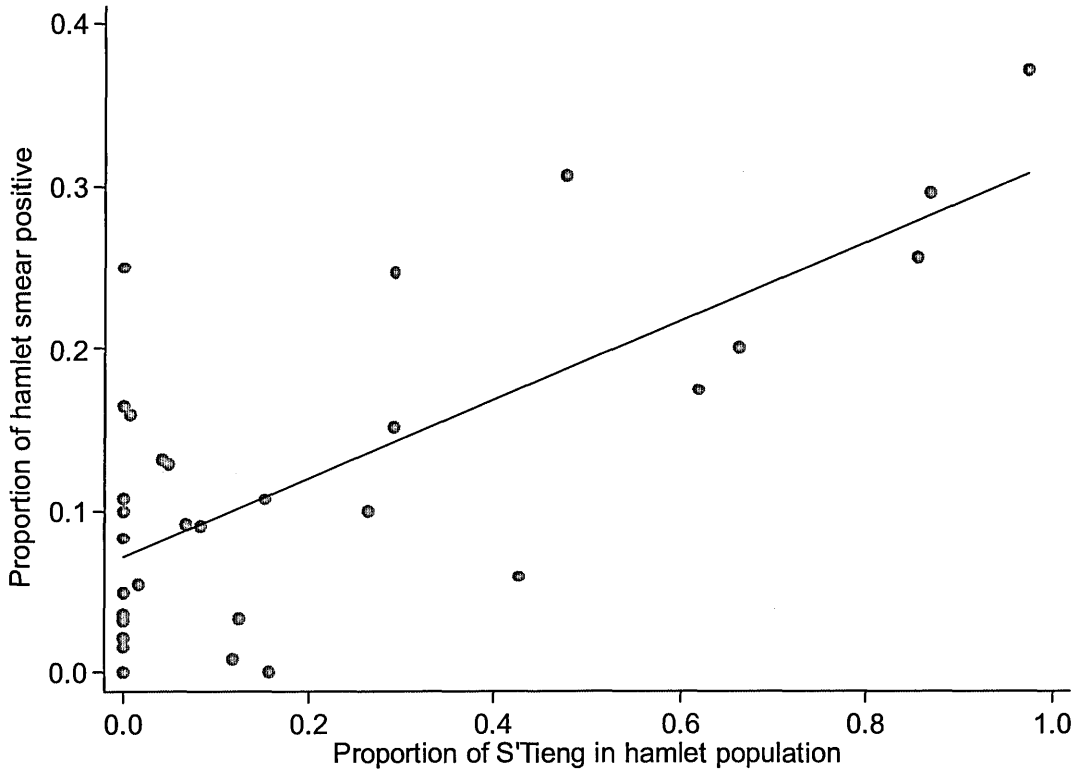


Fig 3.12. Proportion of hamlet sample from S'tiếng ethnic group vs proportion of sample with positive blood smear. Correlation coefficient 0.79, linear regression coefficient 0.24 ($p<0.001$).

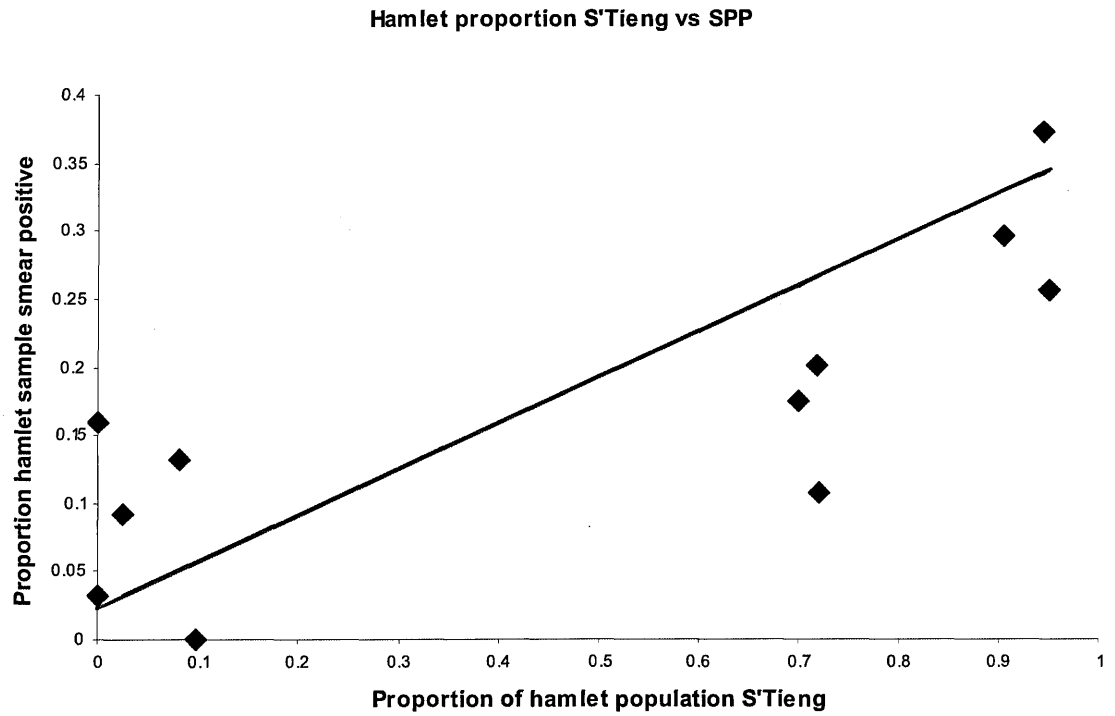


Fig 3.13: Relationship between proportion of hamlet population belonging to the S'tiếng ethnic group and smear positive prevalence in that hamlet (Đắc Ơ commune only). Correlation coefficient 0.79, linear regression coefficient 0.20 ($p<0.001$).

Hamlet	Kinh	S'tiếng	Tày	Nùng	M'Nông	Other
Thôn 3	10% (20)	**24% (78)**	5% (22)			
Thôn 4	17% (18)	**22% (188)**	17% (30)	16% (37)		18% (11)
Thôn6	7% (85)	**29% (17)**				
Thôn 7	0% (21)					
Thôn 9	8% (136)					
Bù Bưng	7% (29)	**33% (191)**				
Bù Cà		38% (186)				
Bù Khơn	17% (12)	**28% (100)**				
Bù Xia	16% (135)					
Đak Lim	2% (53)					
Đak U	13% (177)					
Ấp 4	17% (143)	**20% (75)**				0% (25)
Ấp 1	**2% (49)**	0% (13)	0% (22)	0% (22)		
Cầu 2	**5% (73)**		0% (25)			
Ấp 2		4% (79)	0% (17)	**9% (80)**		
Ấp 3	4% (103)					
Ấp 6	23% (87)	**29% (45)**				23% (22)
Suối Binh			1% (69)	**2% (58)**		
Ấp 5	7% (46)	**57% (42)**				
Bù Xia			1% (116)	8% (13)		
Đak Xuyền	5% (44)	0% (11)			**15% (66)**	
Đak La	0% (23)				**14% (90)**	
Đak Ma	9% (94)					
Bù Ghe	**13% (24)**				12% (26)	
Đắc Liên	**29% (24)**				14% (152)	
Bù Oai	7% (124)	**16% (63)**	8% (24)			9% (22)
Đak Nùng	5% (22)				**21% (67)**	
Thống Nhất	0% (41)			0% (26)		
Đang Lang					5% (171)	
Đak Úy	3% (29)	**18% (11)**	0% (35)			

Table 3.20. SPP amongst different ethnic groups by hamlet. Only those ethnic groups with more than 10 individuals in a hamlet sample are included. Highest SPP in a hamlet is in bold for those hamlets with 2 or more ethnic groups with sufficient representatives in the survey. Cells contain percentage smear positive (total number of observations) in hamlet ethnic subsample.

Symptomatology

Demographics

There were unexpected variations in the reported incidences of different symptoms between villages (full breakdown of symptoms and signs by village is presented in table 8, appendix 2). Once again geography and ethnicity are intertwined, and the incidence of sore throat, rigors, chills and headache were higher in the M'Nông (full symptomatology breakdown by ethnic group is presented in table 7, appendix 2). This pattern held for

smear negative individuals. There was no dearth of asymptomatic M'Nông, however, thus the excess incidence of various symptoms was due to the number of polysymptomatic M'Nông. Whether these discrepancies is due to genuine differences in symptomatology or simply reporting and recording variation is difficult to establish. The former is suggested by the higher prevalence of signs, such as jaundice and pharyngitis, in the M'Nông, although the jaundice might potentially be due to a (speculatively) high prevalence of either hepatitis B or alcoholic hepatitis, and criteria for noting pharyngitis might differ between doctors. The latter is suggested by the differences in crude symptom count between villages in Kinh individuals, the only ethnic group to be represented in significant numbers in symptomatic samples from all three villages (table 3.21).

Village	Number of observations	Mean number of symptoms and signs reported
Đắc Ơ	88	2.11
Đồng Tâm	142	1.73
Đak Nhau	34	3.50
Total	264	2.08

Table 3.21. Variation in symptom reporting between villages amongst Kinh subjects

Different medical staff were involved in the survey in different villages, so it's not possible to analyse potential inter-individual differences in recording in isolation. If these differences in symptomatology are real, they may represent variation in invitation strategy between villages or attendance behaviour between ethnic groups, either of which might bias the smear positive prevalence data. Another inconsistency was the reporting of unfeasible headache and chills in preverbal age groups. During the first week of the survey it was clear that auroscopy was only performed in the presence of frank localising symptoms. Exhortations to examine the ears of all children with fever did not lead to a noticeable increase in auroscope use in the subsequent weeks, thus these data should be interpreted with caution.

Symptoms and malaria

The inadequacy of a clinical diagnosis of malaria has long been recognised, so our failure here was, perhaps, predictable. The presence of definite features of an alternative diagnosis has often been used to rule out a diagnosis of malaria fever. Significant

diarrhoea (greater than 5 loose stools per day), evidence of otitis media, and chest signs are usually taken as indicating a non-malarial aetiology of fever. Only 5 of the smear positive individuals in the survey demonstrated one or more of these features, too few to allow any conclusions to be drawn. Including those with diarrhoea but less than 5 stools (total number with any feature 11), clinical pharyngitis (17) or both (21), increases the numbers at the risk of losing specificity. A simple positive symptom score (rigors+chills+splenomegaly +pallor with or without the addition of headache) is normally distributed in these "definitely non-malarial" individuals, indicating either the unreliability of reporting or lack of specificity of these features. More surprising, however, is the finding that the parasitaemia was the same in the definitely negative group compared to other symptomatic smear positive individuals, becoming significantly *higher* when the broader negative symptom definitions were compared to all smear positive individuals (table 3.22). The assumption underlying all previous models of parasitaemia and fever is that the risk of malarial fever increases with increasing parasitaemia. Non-malarial fever may suppress or may not affect an existing parasitaemia, but has not been shown to increase the parasitaemia. In the face of these data, one is reluctant to exclude a malarial aetiology for the fever even in those individuals with "definitely" negative features.

Definition of symptom complex	Symptom complex status	Number	Mean $\log_{10}$ *Pf* parasitaemia/µl	Standard deviation	p value
A: >5 stools/day or abnormal chest signs or ear localising symptoms or signs	Yes	5	3.9	1.6	
	No-febrile	120	4.1	1.0	0.66
	No-all	314	3.7	1.0	0.74
A or any diarrhoea	Yes	11	4.1	1.3	
	No-febrile	114	4.1	1.0	0.91
	No-all	308	3.7	0.9	0.19
A or sore throat	Yes	17	4.2	1.1	
	No-febrile	108	4.0	1.0	0.57
	No-all	302	3.7	0.9	0.04
A or any diarrhoea or sore throat	Yes	21	4.2	1.1	
	No-febrile	104	4.0	1.0	0.43
	No-all	298	3.7	0.9	0.01

Table 3.22: Log_{10} falciparum parasitaemia in individuals with definite non-malarial features

The risk of misattributing a non-malarial fever to malaria increases with the prevalence of asymptomatic parasitaemia. Individual likelihood of asymptomatic parasitaemia depends on age and current and previous exposure. Naive individuals and young children (after the waning of maternally derived passive immunity) are likely to be symptomatic with any patent parasitaemia. A few such individuals may be caught in a survey in the short period between the emergence of parasites in the blood, and the onset of symptoms. As immunity improves with further exposure, the chance of asymptomatic parasitaemia increases, peaking, in high transmission regions, in early to mid childhood. Children in particular can harbour quite significant number of parasites without displaying symptoms, yet without being able to clear these parasites, possibly due to the earlier development of "disease specific" immunity. Whether these effects are related to age or purely due to exposure is unclear, although sufficient exposure is clearly necessary.

Age	Kinh	S'tiêng	Tày	Nùng	M'Nông	Others	Total
<1	0	50% (4)	0	0	50% (2)	0	50% (3)
1	50% (2)	50% (10)	0	0	0	0% (1)	46% (6)
2-4	60% (15)	41% (63)	0	0	38% (13)	0	44% (40)
5-9	56% (34)	23% (91)	100% (2)	0% (1)	44% (25)	67% (3)	35% (55)
10-14	63% (27)	21% (34)	25% (4)	0% (1)	71% (7)	50% (2)	41% (31)
15-19	64% (14)	5% (22)	0	50% (6)	0% (3)	100% (1)	30% (14)
20-29	65% (26)	21% (28)	33% (3)	0% (1)	20% (10)	0% (3)	37% (26)
30-39	71% (21)	0% (18)	50% (2)	33% (3)	17% (6)	0% (1)	35% (18)
40-49	31% (13)	0% (8)	0% (1)	50% (2)	67% (3)	0	26% (7)
50-59	50% (8)	0% (11)	50% (2)	0% (2)	100% (1)	0	25% (6)
60-69	100% (1)	0% (6)	0	0	0	0	14% (1)
70+	0% (2)	25% (4)	0% (1)	0	0% (1)	0	13% (1)
Total	59% (163)	23% (299)	40% (15)	31% (16)	39% (71)	36% (11)	36% (575)
SPP	9.9%	26.48%	3.55%	5.9%	12.2%	8.15%	13.74%

Table 3.23. Breakdown by ethnic group of percentage of smear positive individuals in different age groups reporting fever or being febrile at the time of survey. Figures in brackets are total number of smear positive individuals in that ethnic and age group. Smear positive prevalence for the ethnic group as a whole is included for comparison.

The relationship between age, parasitaemia and measured or reported fever varies between ethnic groups in our sample (table 3.23). The pattern in the S'tiêng conforms to that expected in an area of moderate transmission, with the burden of symptoms peaking in school age children, and declining through adulthood such that individuals over 30 are

rarely symptomatic. Parasitaemic S'tiếng with measured or reported fever were significantly younger than both similar individuals in other ethnic groups (mean age 7.8 years vs 17.9 years, $p<0.0001$) and asymptomatic S'tiếng (mean age 7.8 vs 16.6 years, $p=0.0001$). This relationship did not reach significance for any other ethnic group. The pattern in the Kinh resembles that in conditions of much lower transmission, with a similarly high proportion of smear positive individuals exhibiting symptoms regardless of age. The flat curve of fever rate against age in parasitaemic Kinh is somewhat surprising given the non-trivial SPP of 9.9%, and suggests that this may not be a representative sample in terms of either symptomatology or smear positivity. A significant number of Kinh living in this area are immigrants from the north of Vietnam, and an alternative explanation might be the relative lack of immunity amongst migrants from provinces with a low malaria prevalence, who might also be poorer and more likely to attend the survey. We did not gather information on migrant status in this survey. Neither do other ethnic groups behave quite as expected. Whilst the numbers of Tày, Nùng and other ethnic groups are too small to comment on age related symptomatology, one might have expected a higher overall fever rate given the relatively low SPP. Similarly the M'Nông, who were expected, a priori, to resemble the S'tiếng, demonstrate more symptoms than might be expected from their overall smear positive prevalence. The excess of symptoms and signs amongst the M'Nông independent of parasitaemia was noted above, and it seems likely that the same phenomenon, whether over reporting or a different sampling strategy, is responsible here.

The problem of attributing fever to malaria in the face of a high prevalence of asymptomatic parasitaemia has taxed malariologists for decades. A number of approaches have been developed which may be useful in this context of dubious clinical data. The first approach uses logistic regression models of fever risk on various functions of parasitaemia, extended to include an age covariate, to generate a malaria attributable fever fraction, against which to test other case definitions. The second approach also utilises

logistic regression of fever on parasitaemia and age, then examines the resulting model for discontinuities, suggesting levels of parasitaemia that constitute "pyrogenic thresholds" at different ages. Whilst these methods may prove useful in the longer term, it is clear from the data presented in the preceding paragraph that any model of the relationship between fever, parasitaemia and age in this environment must also take account of ethnic group. The current data are therefore too sparse to support a reasonable model of this sort.

Another approach taken in a similarly low transmission region has been to use the geometric mean parasitaemia of those individuals at their chronological threshold of fever (ie those found to be pyrexial at the time of examination, but who denied a history of fever) to represent the parasitaemic "pyrogenic threshold". Applying this to our population as a whole (fig 3.14), would indicate a threshold of 11,000 parasites/mcl, close to the 10,000 parasites/μl often used as a malaria defining cut off in clinical malaria studies. The same caveats about age and ethnic group apply to this estimate, although this rather more empirically derived value may be more robust. Only 11 of the 91 adults with parasitaemia without fever had parasite counts greater than 11,000/mcl, however 38 of the 133 children with asymptomatic parasitaemia had higher counts. Repeating this exercise for different age groups (fig 3.15) or ethnic groups (fig 3.16) rapidly runs into difficulty with small numbers in each category, even using fewer age group categories. Although it appears that the cut off value generated in this fashion would be lower for the Kinh than the S'tiếng, breaking this down further reveals that the individuals in the critical category (febrile at the time of the survey with no history of fever) are drawn from different age groups in the different ethnic groups, and that data is sparse (selected groups shown in fig 3.17).
A final, pragmatic approach is to postulate that in a low transmission environment, any fever in the presence of parasitaemia can be regarded as due to malaria. This position is supported by the observation that in certain conditions, a great majority of afebrile individuals found to be parasitaemic in a cross sectional sample go on to develop symptoms in the ensuing days. Based on the data from this survey sample, it is likely that

this model would be valid for Kinh, Tày, Nùng and possibly M'Nông, but not for the S'tiếng. In the absence of better data, however, it suffers from fewer assumptions than other systems, and will be used as our working model.

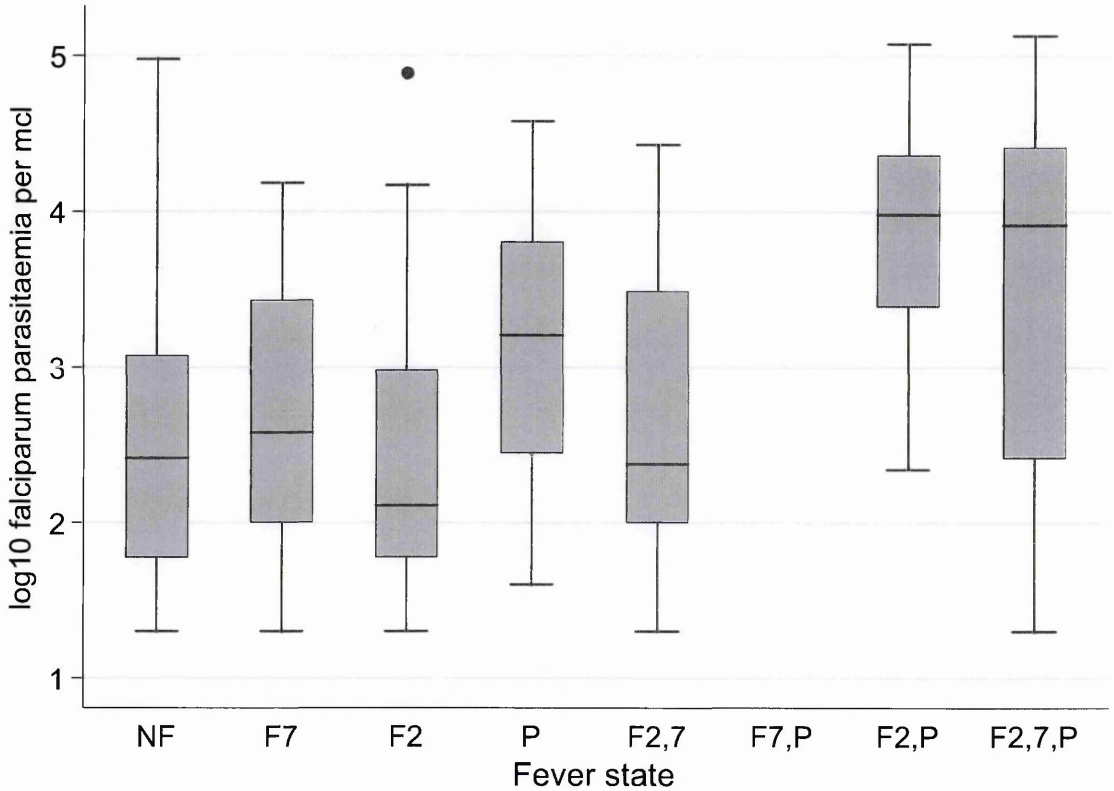


Fig 3.14. Log parasitaemia vs fever state. NF-no fever, F2 – reported fever in previous 2 days, F7 – reported fever in previous 7 days, P – pyrexial at time of survey, and combinations thereof.

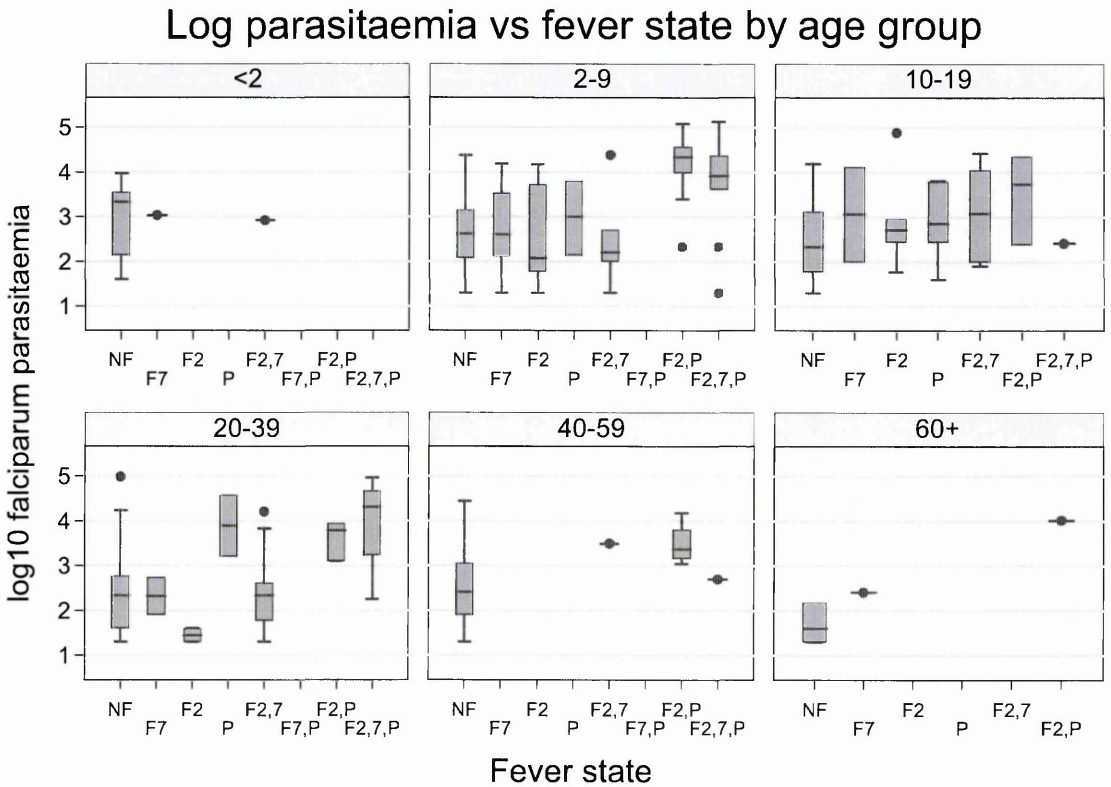


Fig 3.15. Log parasitaemia vs fever state by age group. Fever state codes as fig 3.14.

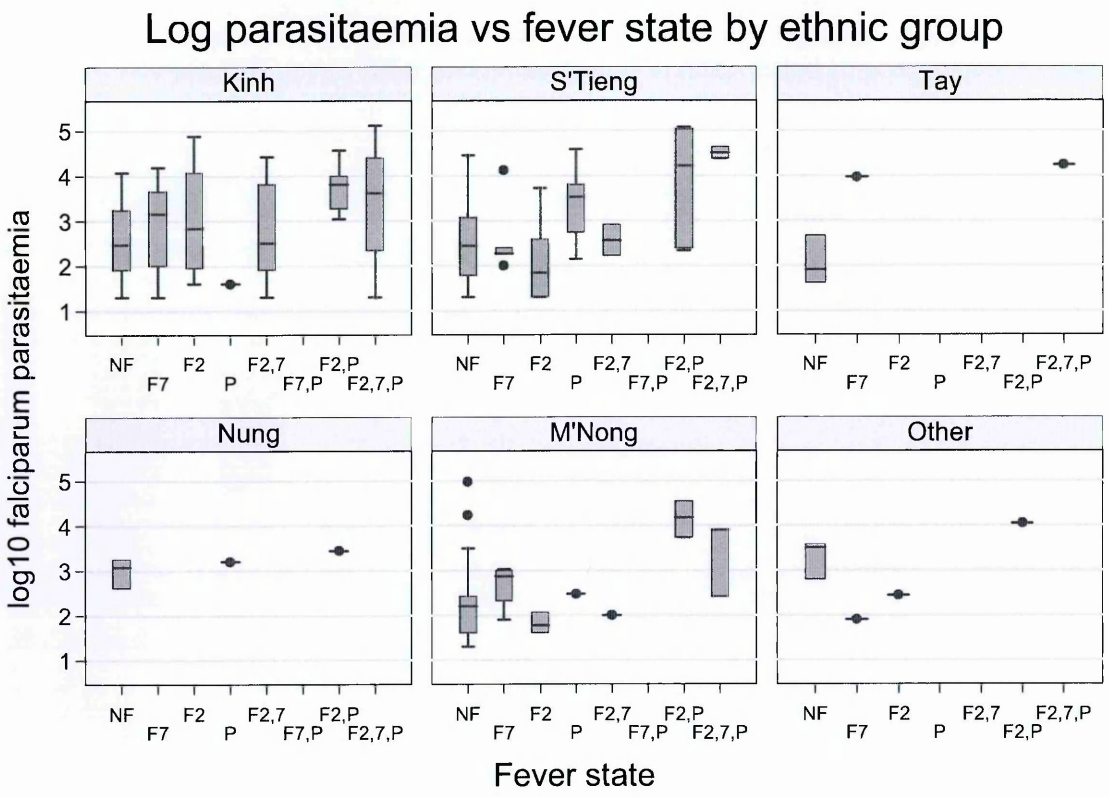


Fig 3.16. Log parasitaemia vs fever state by ethnic group. NF-no fever, F2 – reported fever in previous 2 days, F7 – reported fever in previous 7 days, P – pyrexial at time of survey, and combinations thereof.

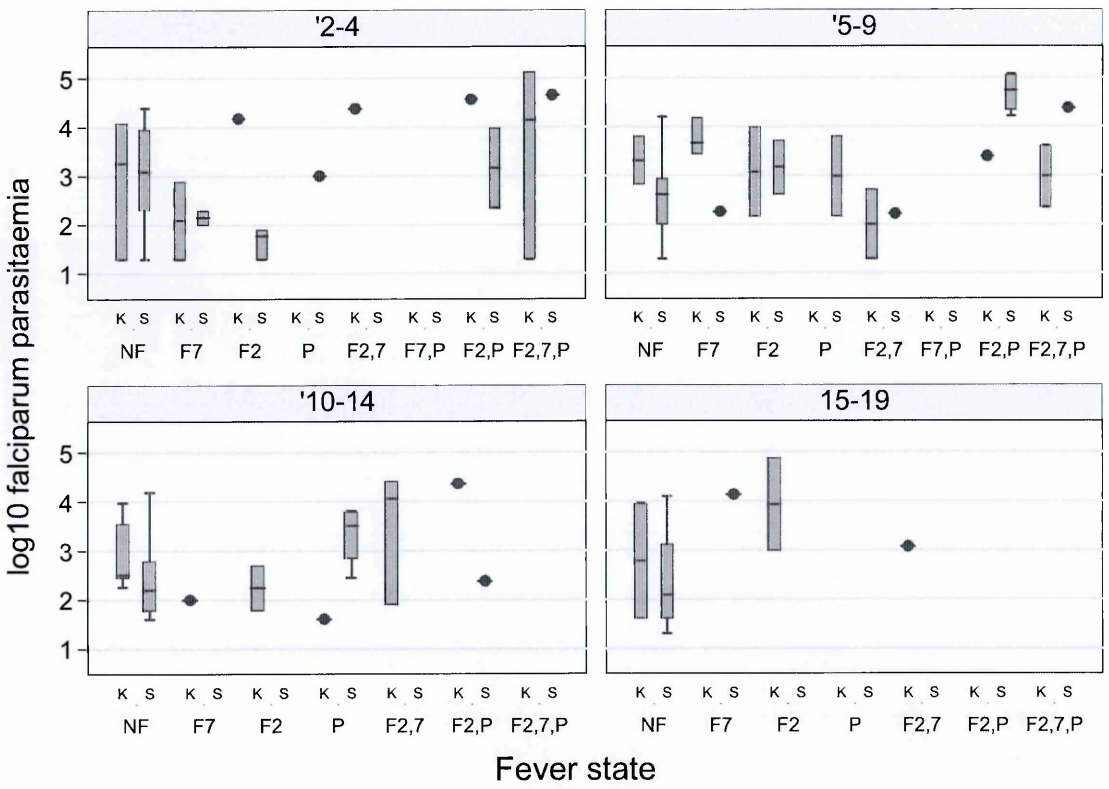


Fig 3.17. Log parasitaemia vs fever state for Kinh and S'tiếng individuals for selected age groups. Fever state codes as fig 3.16.

Conclusions

Despite the methodological inadequacies of this survey, three significant results stand out: the S'tiếng appear to suffer higher malaria transmission than other ethnic groups, as evidenced by the younger, and more pronounced, peak of parasite prevalence, and the steeper decline of symptoms with increasing age, in addition to the generally higher parasite prevalence; the S'tiếng have a very high gene frequency of HbE; and the prevalence of parasitaemia varies considerably over quite short distances.

These observations carried obvious consequences for future studies: it would be necessary to conduct further surveys in a number of different hamlets in different villages to confirm the geographic variation and attempt to elucidate any patterns therein; gathering information on migrant status and bednet use should be performed in further surveys; a knowledge, attitudes and practice study (KAP) comparing the malaria risk behaviour of S'tiếng and other ethnic groups, in particular the Kinh, was essential to try and explain the difference in transmission; and studies comparing entomological indices between high and low transmission hamlets, and between population centres of the different ethnic groups, would be extremely helpful, if not essential, in understanding the apparent variation in transmission.

Additional points to emerge which have implications for the conduct of these studies were the significant number of individuals attending the survey from the same family, necessitating a better individual identification system; the unexpectedly high symptom rate given the Kinh smear positive prevalence, suggesting we should gather information on migrant status in future surveys; and the difficulty in modelling the relationship between parasitaemia and symptoms rendering a longitudinal study to examine the relationship between the two highly desirable. It became clear that any longitudinal study would take time to organise, so as part of the subsequent cross sectional survey we revisited all the smear positive individuals after the survey to question them on the development of

symptoms, and the results are presented in chapter 4, together with details of other subsequent surveys.

The higher smear positive prevalence in Đắc Ơ, together with the high HbE prevalence in the S'tiếng and the higher concentration of S'tiếng in Phước Long, made Phước Long district the obvious site for future studies.

Chapter 4 – Haemoglobinopathy Prevalence

Introduction

Determining the prevalence of red cell disorders in the study population was essential to ascertain ethnic differences in HbE, ensure there was sufficient HbE to offer at least the possibility of a result from the case control study, and document the prevalence of other disorders, in particular α-thalassaemia, which might potentially interact with HbE. Assaying a large number of individuals also established robust estimates of haemoglobinopathy prevalences in the study area, with which the matched controls in the case control study could be compared.

The published data on haemoglobinopathies in Vietnam have been reviewed in chapter 2: the majority Kinh and undifferentiated Vietnamese subjects assumed to be Kinh had a low (1-5%) prevalence of HbE and a low to moderate (1-3%) prevalence of β-thalassaemia. Some ethnic minority groups, particularly from the north, demonstrated high prevalences of β-thalassaemia, whilst others (in particular the S'tiếng, Khmer and Ê Đê) had very high prevalences of HbE. No systematic surveys of α-thalassaemia had been undertaken.

The HPLC analysis was conducted in Professor John Clegg's laboratory in Oxford, and I had no part in conducting the assays or interpreting the results. This was a deliberate attempt to blind the investigators in Vietnam from the haemoglobin type of the study subjects. The majority of the HPLC assays were conducted by Katie Miles, and latterly by Angela Allen. The majority of the α thalassaemia PCR's (including DNA extraction) were performed by Angela Allen, some 250 having been completed by Katie Miles. DNA from the samples from the second survey was extracted by Raveen Basran, as part of her own PhD project. A small number of the α thalassaemia assays on survey samples were conducted in Vietnam by Ty Hang and Ngoc Quyen, who also extracted the DNA. My only involvement in the laboratory work was aliquoting many of the samples, extracting

DNA from some of the samples, and supervising the lab work in Vietnam on the basis of experience gained in John Clegg's lab at the beginning of the project.

Additional methods

Details of the survey designs and laboratory techniques have been given in chapter 2. This chapter includes data from three previously unpublished surveys conducted by other members of the unit in Vietnam, the designs of which are briefly described here.

Two surveys had been conducted in 1999, one in Bù Đăng district of Bình Phước province, the other in Khánh Hòa province in south central Vietnam. The terrain and ethnography of Bù Đăng have already been described. Khánh Hiệp and Khánh Bình communes in Khánh Hòa province are remote areas in the intermediate foothills of the central highlands, lying between 400 and 800m above sea level. The local population is predominantly of the Rac Lay and Ê Đê ethnic groups, with pockets of mostly Kinh immigrants. Many inhabited areas lie close to primary or secondary forest. The Bù Đăng survey was conducted with the assistance of IMPE, and the Khánh Hòa surveys were carried out in conjunction with NIMPE and the Khánh Hòa Provincial Malaria Control Programme, Nha Trang. The primary objective of these surveys was to assess the prevalence of malaria in the study populations. A thick and thin blood smear and capillary blood samples were taken into locally produced, heparinised 75µl glass capillary tubes and stored at 4°C before being transported to Oxford to be analysed by HPLC as described. The third of the unpublished surveys was conducted in 2001 amongst students from universities in Hồ Chí Minh City recruited as a control group for genetic studies being undertaken in the unit at that time, predominantly on G6PD. The students were drawn from a wide region of south and central Vietnam, although the largest single group were from HCMC and its environs. They were all Kinh. The G6PD status is the only data from this survey presented here. This was determined by the semi-quantitative methylene blue test, details of which are given in appendix 3. The test relies on the different absorptive spectra of the

reduced and oxidised states of methylene blue. The deep blue of the natural oxidised form disappears on reduction: blood from G6PD replete subjects completely decolourises the test sample, whilst no colour change indicates completely deficient individuals, and samples from partially deficient individuals result in intermediate hues.

Results:

In total 4908 Kinh, 4326 S'tiếng, 538 Tày, 413 Nùng, 524 M'Nông, 501 Rac Lay, 266 Ê Đê and 108 Dao underwent haemoglobin typing by HPLC. All the Rac Lay and Ê Đê subjects and 248 of the Kinh subjects are from Khánh Hòa, whilst 591 Kinh, 125 Tày, 54 Nùng, 52 S'tiếng and 98 of the Dao subjects are from the 1999 Bù Đăng survey. The remainder comprise all samples from the 3 surveys conducted in 2000, together with a tranche from the March 2001 survey. The results are presented in table 4.3.

The gene frequency of HbE was high in the S'tiếng (0.362), the M'Nông (0.234), the Ê Đê (0.314) and the Rac Lay (0.144) ethnic groups, and low in the Kinh (0.021), Tày (0.018), Nùng (0.009) and Dao (0). The beta E gene is in Hardy-Weinburg equilibrium in all the populations with a high prevalence, although there was a non-significant dearth of heterozygotes in the S'tiếng (observed vs expected: AA 1756 vs 1728, AE 1933 vs 1989, EE 600 vs 572). There was a significant excess of homozygotes in the Kinh, although the absolute magnitude of deviation from expected values was small (observed vs expected: AA 4651 vs 4647, AE 197 vs 204, EE 6 vs 2).

The prevalence of β-thalassaemia was low amongst the ethnic groups in which HbE was common: 0.5% in the S'tiếng, 0.2% in the M'Nông, 0% in the Rac Lay and 1.5% in the Ê Đê (although this represents just 4 individuals in a sample of 270). Whilst the Tày (6.5%), Nùng (10.4%) and Dao (9.3%) demonstrated moderate and high prevalences of β-thalassaemia trait, we found only 1.6% of Kinh were β-thalassaemia heterozygotes.

The prevalence of HbE/β thalassaemia compound heterozygotes is not reported. It became clear as samples from the second and subsequent surveys were being processed that levels of HbA2/HbE were not as high as might be anticipated in HbE carriers (table 4.1). Proportions of HbF also appeared higher than expected in older children and adults. Isoelectric focusing analysis of the haemoglobin eluting in the HbF window suggested the presence of degradation products, rather than true HbF. The diagnosis of HbE/β thalassaemia relies on the presence of only HbE and HbF, the latter usually comprising at least 15% of the total haemoglobin, depending on the accompanying β thalassaemia mutation (Weatherall et al. 2001a). The false elevation of HbF cast doubt on the reliability of the assay, when used in these samples, to diagnose HbE/β thalassaemia. Although extremely important on both individual and population health levels, the absence of this data does little to affect estimates of prevalence of either trait alone, and is very unlikely to affect any other studies, as the numbers will be extremely small. The March 2000 survey seemed to give the most reliable results, and amongst the S'tiếng in this survey there were 5 individuals over the age of 2 with HbA2/E and HbF levels suggestive of HbE/β thalassaemia, against an expected number of 6 based on the calculated gene frequencies. More concerning, in terms of this programme, was the possibility that the depressed levels of HbA2/E might affect our ability to robustly attribute HbE genotype. Figs 4.1 suggests that this is not likely, as there is a clearly tri-modal distribution in each survey. Individuals with HbA2 falling between the bulk of results for HbE heterozygotes and that for HbE homozygotes were not classified. The impact of the depressed HbA2 on our ability to diagnose β thalassaemia trait was slightly more worrying. Table 4.2 illustrates that the prevalence data is likely to be robust, as it's dominated by the more reliable March 2000 survey, and that, with the exception of the December 2000 survey, the estimates of β thalassaemia prevalence are consistent across surveys. Given the very low prevalence of β thalassaemia in the ethnic groups with high prevalence of HbE, misattribution of β thalassaemia status will not affect the association studies.

Survey	Normal	β thalassaemia trait	Heterozygous HbE	Homozygous HbE
March 20000	2.7 +/- 1.7	5.3 +/- 1.2	27 +/- 4.0	87 +/- 9.9
August 2000	2.2 +/- 0.4	4.4 +/- 0.7	20 +/- 3.5	70 +/- 5.5
December 2000	2.2 +/- 0.6	4.9 +/- 2.2	17 +/- 3.2	50 +/- 8.6
Mar 2001	2.2 +/- 1.4	4.4 +/- 1.9	19 +/- 4.0	62 +/- 12.9
December 2001	1.9 +/- 0.5	4.2 +/- 1.2	17 +/- 3.0	56 +/- 6.0

Table 4.1. Mean +/- sd HbA2 for different genotypes in the different surveys.

Survey	Kinh	S'tiếng	Tày	Nùng
March 2000	1.9% (1644)	0.2% (1124)	7.8% (423)	7.0% (271)
August 2000	1.5% (1038)	0 (490)	0 (32)	4.6% (65)
December 2000	2.3% (1158)	1.3% (1193)	6.3% (80)	13.0% (100)
March 2001	0.5% (1005)	0.2% (823)	14.3% (21)	10.5% (19)
December 2001	0 (301)	0.2% (954)	3.7% (54)	11.8% (76)

Table 4.2. Estimates of β thalassaemia trait by ethnic group and survey. Cell contents are percentage carrying β thalassaemia (total number of observations).

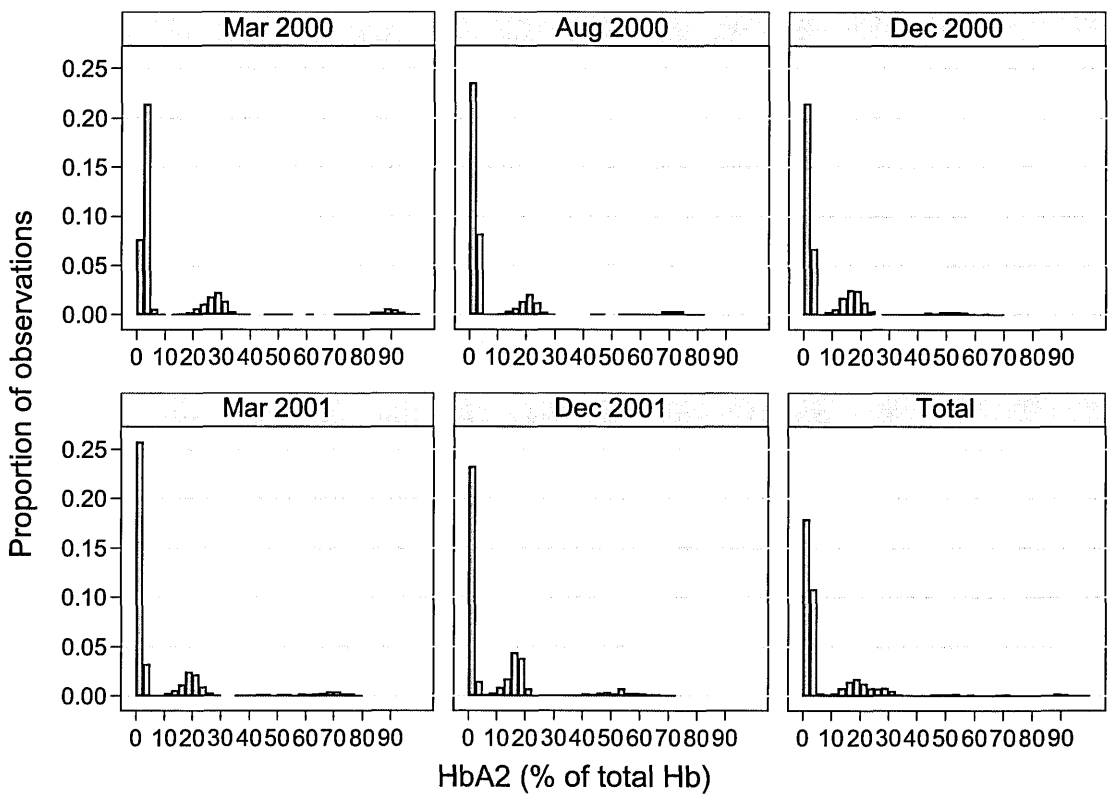


Fig 4.1a. Distribution of HbA2 in the different surveys.

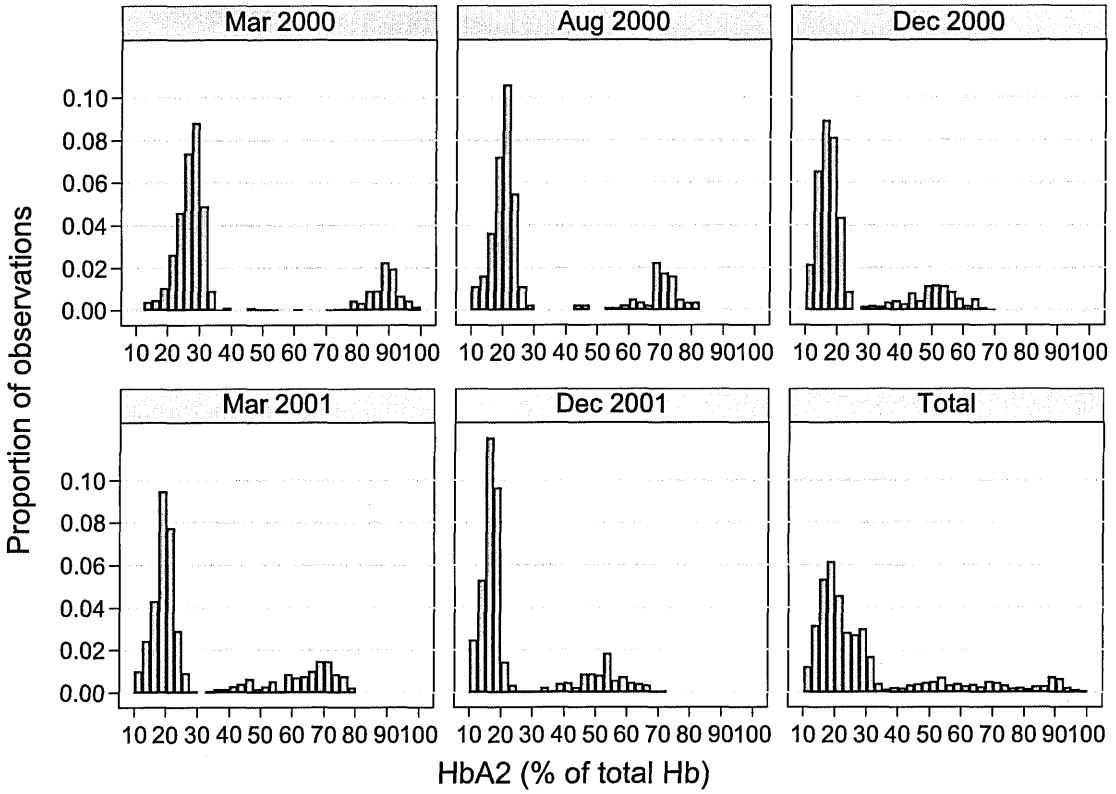


Fig 4.1b. Distribution of HbA2 in the different surveys for individuals with HbA2≥10%.

The problem of degradation of samples was difficult to resolve. It was not prominent in the first survey, and not apparent from the small trial of handling conditions (see Chapter 2). Some time elapsed before the next tranche of samples was sent, and it was only after their analysis that the issue became apparent. Three aspects of the sample handling were considered as potential culprits: storage conditions in the field prior to freezing at HTD, centrifugation (undertaken as part of sample maximisation), and duration of storage at -20°C. The pilot study had not suggested that the first of these possibilities was likely to affect the results, although it was carried out with aliquots of the same HbAA donor. Attempts to improve refrigeration in the field appeared to have little effect on the results. One of the main differences between the first and subsequent survey sample handling was the centrifugation and aliquoting of the samples. The December 2000 samples were also shipped whole, however, and give amongst the worst results. There were delays of up to 2 years in transporting the samples to Oxford for analysis, and this remains the most likely cause of the degradation. Unfortunately much of this delay was

not amenable to amelioration in the ever shifting politics of sample export from Vietnam. It would have been completely unfeasible to return to study subjects and take a further sample. Not only had considerable time elapsed since the first blood letting, reducing the chances of being able to trace subjects, but the difficulties in identifying individuals has been discussed in Chapter 2. Such a repeat blood letting exercise would also not have been popular with either Vietnamese investigators or the local community, and would only have been worthwhile if it was both feasible and useful.

Rare haemoglobin variants were not reported to the investigators in Vietnam, as they were essentially irrelevant to the hypothesis under examination. Amongst samples for which the raw results in their entirety were entered into a spreadsheet (exclusively from the March and December 2001 surveys), there were 12 samples in which minor peaks eluted outside the HbA, HbA2/E and HbF windows from a total of 3322 samples. Most of these peaks comprised 5-15% of the total haemoglobin. Given the problems with sample degradation, these peaks were not analysed further. This decision was made independently of the investigators in Vietnam, who were not privy to individual results, and would have deferred to the expertise in Oxford. The question of whether the apparent absence of rare variants in such a large survey calls into question the reliability of the bulk of the results is considered below.

Ethnic group & origins / Genotype	Speculative "lowlands" origin				Mon-Khmer speaking highlands populations		Malayo-Polynesian highlands populations	
	Kinh	Dao	Tày	Nùng	S'tiếng	M'Nông	Rac Lay	Ê Đê
AA	4651 (93.9)	98 (91)	482 (89.6)	372 (90.1)	1756 (40.6)	307 (58.6)	364 (72.5)	125 (47)
EA	197 (4.0)	0	19 (3.5)	7 (1.7)	1933 (44.7)	187 (35.7)	130 (25.9)	107 (40)
EE	6 (0.1)	0	0	0	600 (13.9)	29 (5.5)	7 (1.4)	30 (11)
Aβ	54 (1.1)	10 (9)	37 (6.9)	34 (8.2)	37 (0.9)	1 (0.2)	0	4 (2)
Number of subjects	4908	108	538	413	4326	524	501	266

Table 4.3. Beta globin abnormalities in the different ethnic groups. Number of subjects (percentage).

α genotype / Ethnic group	Number assayed	$\alpha_{3.7}$ heterozygotes	α_{CS} heterozygotes	α_{SEA} heterozygotes	$\alpha_{3.7}$ homozygotes	α_{CS} homozygotes	$\alpha_{3.7}/\alpha_{SEA}$ compound heterozygotes	$\alpha_{3.7}/\alpha_{CS}$ compound heterozygotes
Kinh	302	8	0	11	0	0	0	0
S'tiếng	983	232 (948)	34 (969)	54 (890)	78 (948)	14 (975)	10 (948)	11 (957)
Tày Nùng	109	9	0	2	0	0	0	0

Table 4.4. α-thalassaemia genotypes found in Kinh and S'tiếng subjects. See text for gene frequencies. Cell contents are number of subjects carrying that genotype (total number of subjects genotyped for that mutation or combination of mutations).

A randomly selected sample from the March 2000 survey underwent genetic typing for common α-thalassaemia mutations. Further samples were selected on the basis of ethnicity, HbE status and smear result from the August 2000, March 2001 and August 2002 surveys to explore the relationship between malaria, HbE and α-thalassaemia.

The results of molecular analysis of α-thalassaemia in 302 Kinh, 983 S'tiếng and 109 Tày-Nùng are presented in table 4.4. We found no α_{THAI} or α_{FIL} deletions, only one $\alpha_{4.2}$ heterozygote and 3 Hb_{PAKSE} heterozygotes (all S'tiếng). The gene frequency of α_{SEA} was 0.018 amongst the Kinh, 0.009 in the Tày Nùng and 0.034 in the S'tiếng. No Kinh or Tày-Nùng individuals carried $\alpha_{ConstantSpring}$ (α_{CS}), which was present at a moderate frequency of 0.035 amongst the S'tiếng. $\alpha_{3.7}$ was present in both groups, with gene frequencies of 0.013 in the Kinh, 0.041 in the Tày Nùng and 0.215 in the S'tiếng. Overall only half the S'tiếng sample had a full complement of normal alpha globin genes.

The frequencies of G6PD deficiency are presented in table 4.5. Fourteen of the 260 Kinh students were deficient (5.4%). Almost as many women as men were deficient (5.1% vs 5.6%), casting some doubt on the validity of the assay. It is not always clear from the results which subjects were partially, and which completely, deficient. The formazan ring rapid test yielded 6/159 deficient males amongst the Kinh (3.7%), and 22/188 (11.7%) in the S'tiếng. We also found that 6.1% (14/228) of S'tiếng females were deficient by this method, however, and in view of other surveys suggesting a much lower prevalence of G6PD deficiency amongst the S'tiếng, we undertook definitive quantitative testing in 198 S'tiếng males. This proved methodologically challenging: samples must be tested within a few hours of being drawn, necessitating transport of a spectrophotometer to Phuoc Long District Hospital. Despite the use of voltage stabilisers and a UPS, absorption readings tended to be quite erratic. An attempt was made to quantify this problem by reading a blank after each sample. These readings varied widely, casting some doubt on the validity of the measurements. Limiting analysis to the subset of data points with

absolute post measurement blank values<0.01 yielded similar estimates of prevalence, however. A second issue was how to generate cut-off values for the diagnosis of complete and partial deficiency. Histograms using 0.5IU bins for all values and those with low post measurement blank values are shown in fig 4.2. There is a clear population of deficient individuals with activities <3IU/gHb. The documentation accompanying the methods suggests a lower limit of normal of 4.6IU/gHb, and there would appear to be a second subpopulation with activity values between 4 and 6IU/gHb, although this is less easy to distinguish from the lower tail of the normal population (fig 4.3 – bins of 0.25IU/gHb). Using a cut off of 3 for completely deficient, and 4.6 for partially deficient individuals yields estimates of 8.1% completely deficient and 4.5% partially deficient using all samples (total 12.6%), and 8.6% and 2.5% using samples with low post measurement blank readings only (total 11.1%).

G6PD status & method	Kinh males	Kinh females	S'tiêng males	S'tiêng females
Rapid test deficient	6/159 (3.77%)	N/A	22/188 (11.7%)	14/228 (6.14%)
HCMC students completely deficient	8/161 (4.97%)	5/98 (5.1%)	N/A	N/A
HCMC students partially deficient	1/161 (0.6%)		N/A	N/A
Quantitative deficient (activity<4.6IU/gHb)	N/A	N/A	22/198 (11.1%)	N/A
Total prevalence	15/324 (4.6%)	5/98 (5.1%)	44/386 (11.4%)	14/228 (6.1%)

Table 4.5. Prevalence of G6PD deficiency in the different surveys

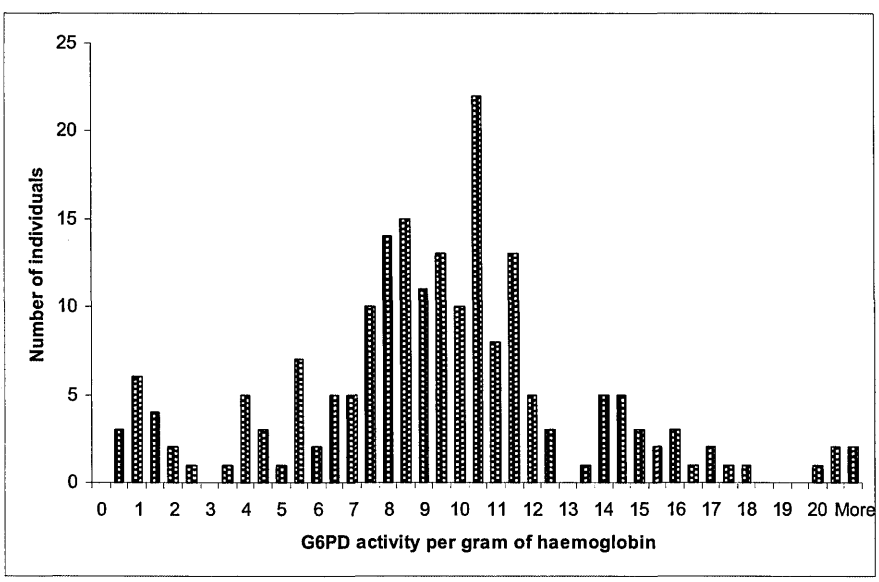


Fig 4.2a. Distribution of G6PD activity in all samples.

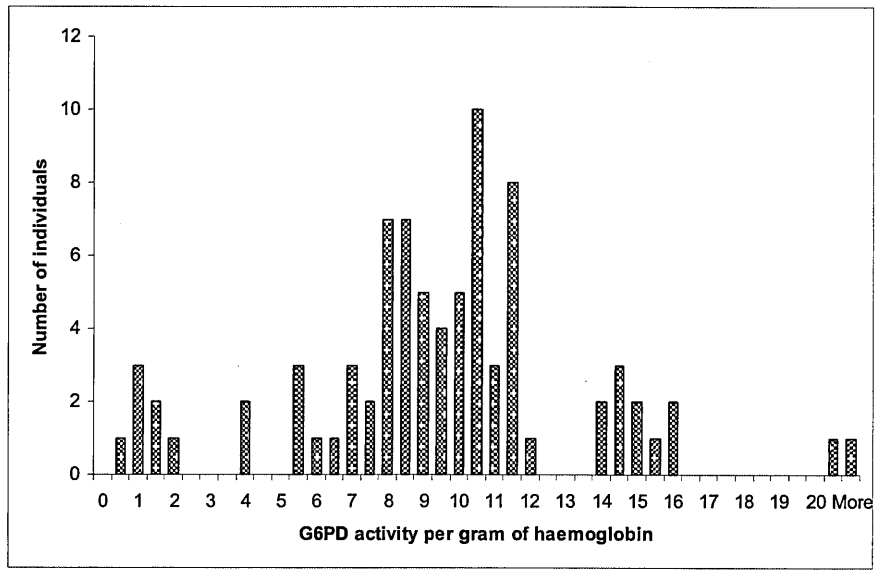


Fig 4.2b. Distribution of G6PD activity in samples with absolute post measurement blank readings<0.01.

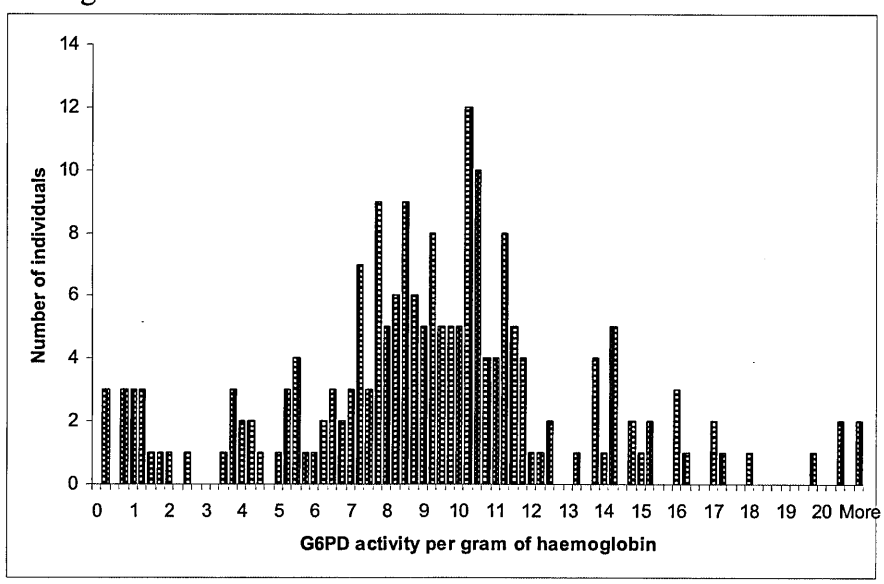


Fig 4.3a. Distribution of G6PD activity in all samples (finer granularity).

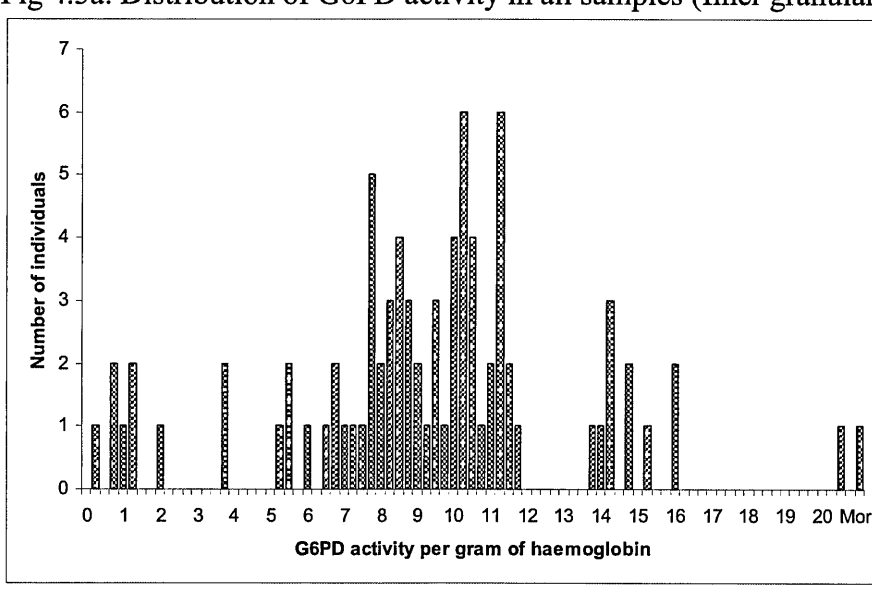


Fig 4.3b. Distribution of G6PD activity in samples with absolute post measurement blank readings<0.01 (finer granularity).

Discussion

Previous surveys of thalassaemia and haemoglobin E prevalence in Southeast Asia have been summarised in chapter 1. The results presented here are broadly in agreement with these studies, and in particular with the consistent findings of high levels of Haemoglobin E and low levels of β-thalassaemia amongst Mon-Khmer speaking ethnic groups, and low levels of HbE and higher levels of β-thalassaemia amongst the Thai-Tày and other populations with putative origins in Southern China. The ethnic minorities included in our study are classified linguistically as Mon-Khmer (the S'tiếng and the M'Nông), Malayo-Polynesian (Rac Lay and Ê Đê), Thai-Tai (the Tày and Nùng) and H'Mong-Dao (the Dao). The Kinh are usually grouped with the southern Chinese minorities. There are dangers inherent in positing deep historical origins on the basis of linguistic categorisation alone, but although the thalassaemias have probably been subject to positive selective pressure from malaria, and thus ethnic differences may reflect different historical malaria experiences, the validity of these broad ethnic divisions would appear to be confirmed by this genetic data. The high prevalence of HbE among the Malayo-Polynesian speakers is interesting. These groups are assumed to be "back migrants" from island Southeast Asia, a region in which HbE does exist, but only at low frequencies. Whether the high prevalence in these groups represents a founder effect, selection by malaria, or interbreeding with "Khmer" groups is a question we do not yet have the data to answer. There is no evidence to suggest it represents a separate origin for HbE, and previous such assumptions based on ethnic identity alone (Deka et al. 1988) have been demonstrated to be wrong (Hundrieser et al. 1988a). Another important exception to the ethnolinguistic-genetic concordance is the Tày-Thai language group, which includes the forebearers both of the modern Thais and the Tày and Nùng. Whilst the latter groups, the total population of which number several million, have a high frequency of β-thalassaemia and virtually no HbE, the former have both a moderate prevalence of β-thalassaemia and approximately 15% HbE, suggesting that the genetic make up of modern Thais has diverged significantly

from their ancestors, possibly through extensive interbreeding with Khmer populations during their southerly migration/expansion (although this supposition has been challenged (Poolsuwan 2003)).

Applying the prevalence data presented here to birth rates calculated from ethnic group specific growth rates (generated from 1994 and 1999 population census figures – table 4.6) and population and crude total birth figures from 2001, we estimate that 34 homozygous β-thalassaemic and 129 HbE/β-thalassaemia compound heterozygote babies will be born to Kinh parents every year. Amongst the ethnic minority groups, these estimates would be 79 and 41 for the Tày, 67 and 14 for the Nùng, 86 and 0 for the Dao, 0 and 8 for S'tiếng, 0 and 2 for the M'Nông, 0 for the Rac Lay, and 2 and 42 for the Ê Đê. The facilities to care for β-thalassaemia major patients in Vietnam are limited to Hanoi and Hồ Chí Minh City. Approximately half the Kinh, all the Dao, and the majority of the Tày and Nùng would be expected, on geographical grounds at least, to receive their healthcare in Hanoi, and the remainder of the Kinh and the other ethnic groups in our study to go to HCMC hospitals. There is no central register of thalassaemia patients, but published figures to date include 72 patients seen at The Institute of Child Health in Hanoi between 1981 and 1983 of whom 45 (72%) were HbE/β-thalassaemia compound heterozygotes (Nguyen et al. 1985), and 197 patients seen at the Transfusion and Haematology Hospital in Hồ Chí Minh City between 1991 and 1993, of whom only 32% had HbE/β-thalassaemia (Tran 1995). In the first of these studies, it is not absolutely clear that all 72 are new patients, and the second study refers to caseload rather than new cases. Neither study is clear about the severity of disease in these patients. A comment in a study of HbH disease from ICH in Hanoi describes making a diagnosis of homozygous β-thalassaemia or HbE/β-thalassaemia in 494 children between 1986 and 1993 (Duong et al. 1994). Numbers of new and existing cases were not available from the hospitals treating thalassaemia in HCMC or Hanoi. Patients followed at two HCMC hospitals are reportedly almost all Kinh, although anecdotally hail from areas in which intermarriage between Kinh

and other ethnicities, in particular Khmer, are more common. The apparent negative correlation between prevalences of HbE and β-thalassaemia in any one population might be expected to be reflected in a relatively low number of individuals being affected by HbE/β-thalassaemia, particularly among ethnic minorities. Only one survey of Vietnamese thalassaemia patients has reported ethnicity (Tran 1995): 184/197 children requiring treatment at the Centre for Haematology and Transfusion in HCMC for β-thalassaemia or HbE were Kinh, 10 were Hoa (Chinese), 2 are described as Khor, although this is not a recognised ethnic designation, and 1 was Tày. No study has reported both mutation type and ethnic origin. The high prevalence of β-thalassaemia amongst some minority groups would be expected to lead to a number of cases of β-thalassaemia major, and the predominance of Kinh amongst the case load is more likely to reflect better access to health care, higher rate of reproductive assimilation, and sheer population numbers than absence of disease in minority groups. There is certainly a suggestion here of considerable unmet need.

Ethnic group	1994 census	1999 census	Annual growth rate	Estimated total births 2001
Kinh	59,012	65,796	2.2%	1,200
Tày	1,190	1,478	4.4%	53
Nùng	705	856	4.0%	28
Dao	474	621	5.6%	28
Ê Đê	195	270	6.7%	15
Rac Lay	72	97	6.1%	4.8
M'Nông	67	92	6.5%	4.8
S'tiêng	50	67	6.0%	3.3

Table 4.6. Population by ethnic group in 1994 and 1999 (thousands) with calculated crude annual growth rates over that period

The α-thalassaemia data presented here indicate 265 Kinh and 135 S'tiêng will be born with HbH disease, and 365 Kinh and 8 S'tiêng babies will die from HbBarts hydrops every year. Once again there is little information about the number of individuals with HbH disease currently receiving health care in Vietnam. One published study from ICH in Hanoi referred to 85 cases diagnosed between 1986 and 1993 (Duong et al. 1994), and a laboratory survey of results from 1883 samples received for Hb electrophoresis at the

Transfusion and Haematology Hospital in HCMC between 1995 and 1998 describes finding 180 cases of HbH disease and a further 63 of HbH/Constant Spring (Vu 1999). Once again no data on the current burden of HbH disease were available from the responsible health care institutions, although as HbH disease is not necessarily symptomatic enough to persuade sufferers to seek health care, interpretation of these numbers would be difficult. No data exist on the incidence of HbBarts hydrops or the associated maternal morbidity.

A number of studies have examined the effect of coinheritance of α-thalassaemia on β-thalassaemia disease, but the interaction between α- and β-thalassaemia *traits* has attracted little attention. This is of particular interest given the recent data from Kenya suggesting epistasis between the HbS gene and single gene deletion α thalassaemia mutations (Williams et al. 2005). An appreciation of this relationship is essential, however, in understanding the population genetics of the thalassaemias: if the prevalence of the thalassaemias is due to positive selection by malaria, and the protection against malaria is mediated by effects of globin chain imbalance, how does a single population come to have such high prevalences of both α- and β-thalassaemias? The mitigating effect of α-thalassaemia on clinical β-thalassaemia major might be relevant in populations, such as the Thai, who have a high prevalence of β-thalassaemia mutations associated with disease (which HbE can be if there is also a significant frequency of other β-thalassaemia mutations). This interaction would be unlikely to significantly change the evolutionary fitness of any mutation, however, as it would have only a relatively modest effect in a tiny proportion of the population (homozygotes or compound heterozygotes). This line of argument cannot be evoked at all in ethnic groups which have HbE to the exclusion of β-thalassaemia, such as the S'tiếng. Another potential explanation is that the protection from malaria presumed to be afforded by haemoglobin E is not mediated through its β-thalassaemia phenotype, and is thus independent of α-thalassaemia, which might even

have an additive protective effect. The small number of S'tiếng cases is unlikely to give a useful answer on the question of interactions, however.

The problem of sample degradation in storage, and it's lack of impact on the assignment of HbE genotype, has been discussed above. It has been suggested that the failure to detect rare variant haemoglobins in a survey of this size may also call into question the validity of the assay on this sample set. There are several reasons why this seems unlikely. No similarly large surveys have been conducted in Vietnam for comparison, and those which have been conducted (all discussed in Chapter 1) have not revealed any rare variants. The rare variants which have been reported in Vietnam are haemoglobins Khartoum (Hendy et al. 1999), J-Lome (Prior et al. 1989), Queens (Moo-Penn et al. 1982), F-Bonaire-Ga (Nakatsuji et al. 1982), Woodville (Como et al. 1986) and Clermont-Ferrand (http://globin.bx.psu.edu/cgi-bin/hbvar/query_vars3?mode=output&display_format=page&i=2542). Haemoglobin Khartoum is unstable, and does elute in the D window, so might have been amongst the abnormal D window peaks, but these were not worked through. Haemoglobin J-Lome and Haemoglobin Queens should have been detected if present, although the latter elutes in the S window, so might have been the abnormal peak detected in this window if sufficient had been degraded to reduce the percentage present. Haemoglobin F-Bonaire-Ga is an HbF variant, and would not have been detected in this study even if present, neither would Haemoglobin Clermont-Ferrand, which is not resolvable on the Variant β short HPLC programme. Haemoglobin Woodville elutes in the HbA2 window, and would thus have almost certainly been missed if present amongst the mass of HbE. All these reports, as with most rare haemoglobin variants, have arisen from the investigation of haemoglobinopathy patients or their families, or discovered accidentally as part of investigation of another condition. Thus the denominator is indeterminable, but clearly vast. Large surveys have been carried out in Thailand and China, which might possibly reflect aspects of the genetic backgrounds of the Vietnamese ethnic groups. Most have reported no rare variants (Jaovisidha et al. 2000; Lau et al. 1997;

Liao et al. 2005; Sin et al. 2000; Tanphaichitr et al. 1995), whilst one reported a prevalence of up to 0.25% (Wong et al. 2006), but did not specify the variants found. It should also be emphasised that the documentation of rare haemoglobin variants was not one of the aims of this body of work, and they would have no impact on the studies' conclusions.

Five studies of the prevalence of G6PD deficiency in Vietnam have been published in English. 701 male blood donors and medical students studying in Hồ Chí Minh City were tested by brilliant cresyl blue dye reduction test. Prevalence varied by region of origin: those hailing from the north of Vietnam having a prevalence of 5.8%, whilst 1.9% of those from the centre were deficient, and only 1.4% of those from the south (Le Xuan et al. 1968). Ethnic origins were not specified. This estimate is similar to the 1.3% documented in 354 southern Vietnamese males (Panich et al. 1980), and the 1.8% of 217 Kinh males (from south and central Vietnam) identified by electrophoresis followed by activity assay amongst deficient electrophoretic patterns (Bowman et al. 1971), but lower than the 3.4% demonstrated in another study (Toncheva 1986). A particularly low estimate of 0.5% was obtained with the fluorescent spot test in Kinh schoolboys in northern Vietnam (Verle et al. 2000). The data acquired here are thus higher than most estimates of prevalence amongst southern Kinh males, although the university students came from all regions of south and central Vietnam, and many of the Kinh in Phuoc Long are immigrants from the north. Whether the significant variation in estimates is a sampling effect or indicates population structure amongst the Kinh is difficult to ascertain. The lack of a consistent geographical trend might militate against the latter, but the stratification may operate at a much more local level than the broad regional differentiation used in the published literature. Prevalence among northern ethnic groups ranged from 0.3% in the Mong to 31% in the Muong, with an average of 20% in "foothill" dwelling minorities (Verle et al. 2000). Amongst southern and central ethnic minorities, Bowman found 15.3% of Khmer, 2.3% of Ê Đê and 5.4% of S'tiếng were completely deficient. Once again the Sedang resembled

the Kinh rather than the S'tiếng, with only 0.4% deficient. The prevalence found amongst the S'tiếng in these studies is thus significantly higher than Bowman's.

The moderately high prevalence of G6PD deficiency amongst the S'tiếng completes their extraordinary full house of red cell disorders. The potential for interactions with HbE are, again, interesting. HbE is oxidatively unstable, and HbE cells have been shown to have reduced activity of antioxidant mechanisms. This might result in a synergistic antimalarial effect, but might also make individuals with HbE and G6PD deficiency more prone to haemolysis. Acute haemolysis in response to dietary triggers is reported to be uncommon in G6PD deficient individuals in Southeast Asia (Kitayaporn et al. 1991). Even the most potent antimalarial trigger – primaquine – may not often result in a haemolytic crisis (Buchachart et al. 2001; Myat Phone et al. 1994), although it has definitely been documented (Karwacki et al. 1989; Khoo 1981). Primaquine is included in the national guidelines for the treatment of vivax malaria, although it is not clear how often it is actually used, and is thus likely to be available in the study community. Dapsone, another documented trigger, is unlikely to be locally available. Quinine is contraindicated, and chloroquine has been associated with haemolysis, although appears to be a less potent trigger (Gaetani et al. 1976; Khoo 1981). There is little data on artemisinin derivatives and G6PD deficiency. The S'tiếng bear the brunt of malaria infection in our study sites, so there must be a significant danger of haemolytic crises. The fact that few are reported may indicate that the problem is more potential than real, or might reflect under use of health care facilities by the S'tiếng. Other potential pharmacological triggers likely to be in widespread use in the study site are co-trimoxazole, aspirin and ascorbic acid, with chloramphenicol and the nitrofurans also possible.

The high ratio of female:male deficient individuals is unexpected, although not unprecedented. Le Xuan's 1968 study included 154 inpatients, both male and female, screened with a quantitative method, and found 8.65% of 69 women were deficient

compared to 5.88% of 85 men, although this population did contain anaemic patients, so may not be representative. Few studies have looked for G6PD deficiency in women, but those that have often demonstrate sex ratios of 0.3-0.5 female:1 male (Khan 2004; Monchy et al. 2004; White et al. 1993), or even 1:1 (Brabin et al. 2004), much higher than the ratio expected if only female homozygotes were to be deficient.

Conclusions:

There is tremendous heterogeneity in the prevalence of HbE and other red cell disorders in Vietnam. The S'tiếng and other "Khmer" ethnic groups have a high prevalence of HbE, as do Malayo-polynesian speaking peoples. Why HbE should be so frequent in two apparently separate genetic pools requires beta globin haplotype analysis, possibly augmented by mitochondrial DNA or whole genome SNP surveys to delineate the genetic origins of these peoples. Ethnic minorities originally from the north of Vietnam and southern China have little HbE. The majority Kinh have low levels of both HbE and β thalassaemia. All ethnic groups studied have at least a moderate prevalence of α-thalassaemias, the prevalent mutations being α_{SEA} and $\alpha_{3.7}$. The S'tiếng have a high prevalence of $\alpha_{3.7}$.

Our prevalence data suggest a significant unmet need for the prevention and treatment of thalassaemias in Vietnam, particularly among ethnic minority groups. The absence of any sort of systematic data collection on the incidence and case load of thalassaemias hampers further assessment of the public health burden.

The Kinh and S'tiếng both have moderate frequencies of G6PD deficiency, with relatively high ratios of affected females to affected males. Mutation analysis of affected individuals are necessary to understand this phenomenon.

The existence of high levels of both α-thalassaemia and Haemoglobin E, and moderate levels of G6PD deficiency, in a single ethnic group raises interesting questions about the

population genetics of the thalassaemias, answers to which may emerge from the malariometric surveys and case control study.

Chapter 5 – Malaria epidemiology

Introduction

The initial survey yielded a number of results which needed to be confirmed: the geographical microheterogeneity in malaria prevalence, the variation between ethnic groups, and the relatively low spleen rate amongst groups with moderate smear positive prevalence being examples. There was an obvious requirement to examine the pattern of malaria over time, and a desire to conduct at least one post survey follow up exercise to gather more data on symptomatology, which had, disappointingly, not emerged from the first survey. The methodological issues identified in the survey needed to be addressed, in an attempt to obtain a sample representative of the population. The census and survey stratagems have been discussed in chapter 2.

Phước Long district had been selected as the location of future studies on the basis of the first survey, and the three most northerly communes in the district comprised the sampling frame for these surveys, as discussed in chapter 2.

Most of the data presented in this chapter pertains to the surveys conducted between August 2000 and April 2003, but where data from the first survey is included this is noted.

This chapter inevitably includes data culminating from the efforts of many field workers. Those involved in more than one survey are detailed in the declaration. In addition to those mentioned in Chapter 3, the following individuals played a major role: Drs Triết, Thái and Khoa were vital to the practical organisation of the surveys from the December 2001 survey onwards. Miss Nguyệt and Tam assisted Miss Ly with reading the smears in the latter stages of the study, as Miss Kim and Điệp moved to other projects. Miss Tâm helped with data entry for the later surveys. The census was performed by the YTTB at the behest of Dr Hiền. My role was to design the surveys and study forms, decide on sampling methods and sample handling, supervise the execution of the surveys, with some

occasional hands on involvement, design and program the databases, enter some data, clean, review and analyse the data.

Sample demographics and sampling success

A total of 16,343 usable observations were made in 11,452 individuals from 3258 households. A breakdown by survey and commune is given in table 5.1. The population pyramids for individual surveys are given in fig 4, appendix 2, with a composite shown in fig 5.1, together with the population data. Fig 5.2 separates the subjects from different survey designs. The population data is derived from national age and sex distribution for relevant ethnic groups combined in the proportions in which different ethnic groups were present in sampled hamlets according to the study census. The ethnic composition of each survey sample is detailed in table 5.2, with official and census data listed for comparison.

The survey sampling methods differed slightly, as set out in Chapter 2. The August 2000 survey was essentially open to all comers. Specific whole families, chosen locally, were invited to join the survey in December 2000, March 2001 and December 2001. Specific individuals, again chosen locally, were invited in the 2002 surveys, and specific individuals, randomly chosen centrally, in the 2003 surveys.

Commune	Population	Aug 00	Dec 00	Mar 01	Dec 01	Apr 02	Aug 02	Dec 02	Apr 03	Total
Đắc Ơ	8410	823	1,414	554	552	483	520	507	277	5,130
Đức Hạnh	18,789	827	1,574	648	586	596	656	639	348	5,874
Đa Kia	14,041	822	1,638	639	439	629	490	456	226	5,339
Total	41,240	2,472	4,626	1,841	1,577	1,708	1,666	1,602	851	16,343

Table 5.1. Sample sizes by survey number and commune.

Survey:	Aug00	Dec00	Mar01	Dec01	Apr02	Aug02	Dec02	Apr03	Total	Official	Census	Included
Kinh	61	49	20	31	28	27	29	28	38	63	65*	51*
S'tiêng	33	45	73	62	64	67	65	68	55	32	35	49
Tày	2	2	3	4	2	2	3	1	3	1	*	*
Nùng	4	3	4	2	4	3	2	1	3	3	*	*
Other	<1	1	<1	1	1	1	1	1	1	1	*	*
Total number	2,469	4,591	1,840	1,576	1,708	1,666	1,602	851	16,303			

Table 5.2. Ethnic composition of survey samples. All figures are percentages except total number. *Kinh and all non-S'tiêng ethnic groups (limited ethnic data available from census).

Assessing the completeness of the family samples is slightly difficult due to the inherent inaccuracy of the census list. On the basis of the list, however, 43% of families invited were completely sampled, 21% were missing one family member, 13% missing two and 14% missing three or more family members. In 9% of household observations the number of family members attending was greater than the number documented in the census list.

A number of features of the sample are apparent from the demographic data. Firstly, Đắc Ơ is over represented. This was an unavoidable consequence of the practicalities of the survey, with a week being spent in each commune: a number of hamlets were dropped from the sampling frame following the December 2000 survey in order to effect the sample size reduction desired to reduce the workload of the survey teams and hence increase accuracy, as the hamlet sample size would otherwise have shrunk to just a few families. None of the Đắc Ơ hamlets were excluded, however, and the sample size is commensurate with the resultant population (data shown in third column of table 5.1). As official population figures were not available at the hamlet level, this last set of data is derived from the study census. Malaria prevalence was generally slightly higher in Đắc Ơ than the other communes, thus the survey estimates of population SPP for the region as a whole are likely to be too high. The proportion of the samples derived from each commune are fairly constant between surveys, however, so the bias in favour of Đắc Ơ is not expected to affect any of the temporal trends or other associations, and analyses will not usually be stratified by commune.

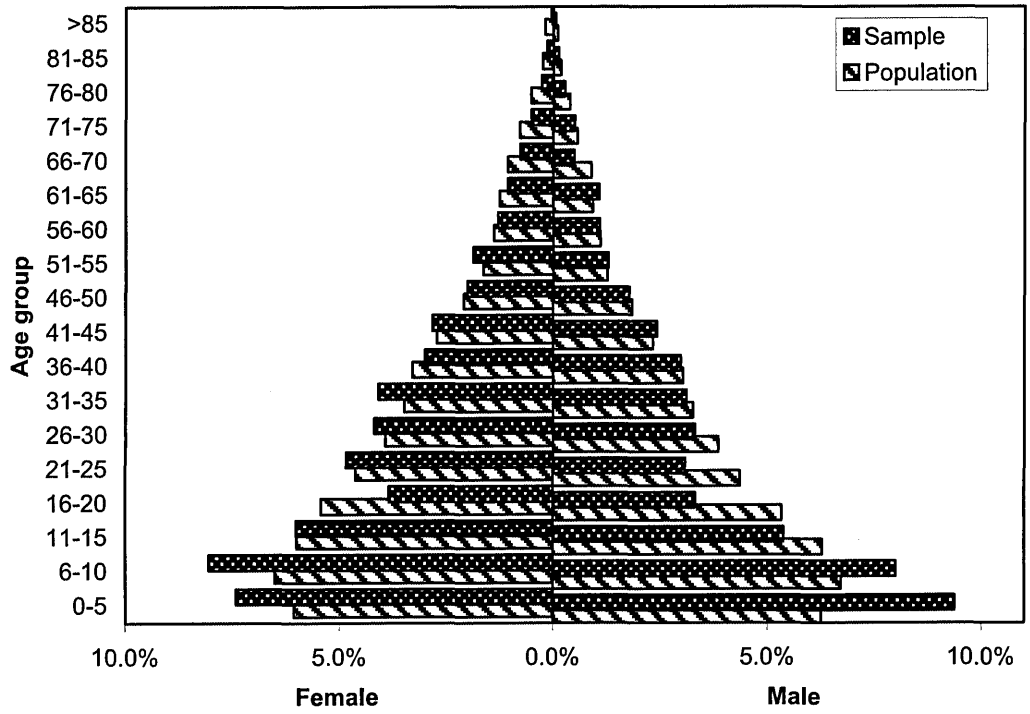


Fig 5.1. Cumulative sample age and sex compared to estimated population age and sex

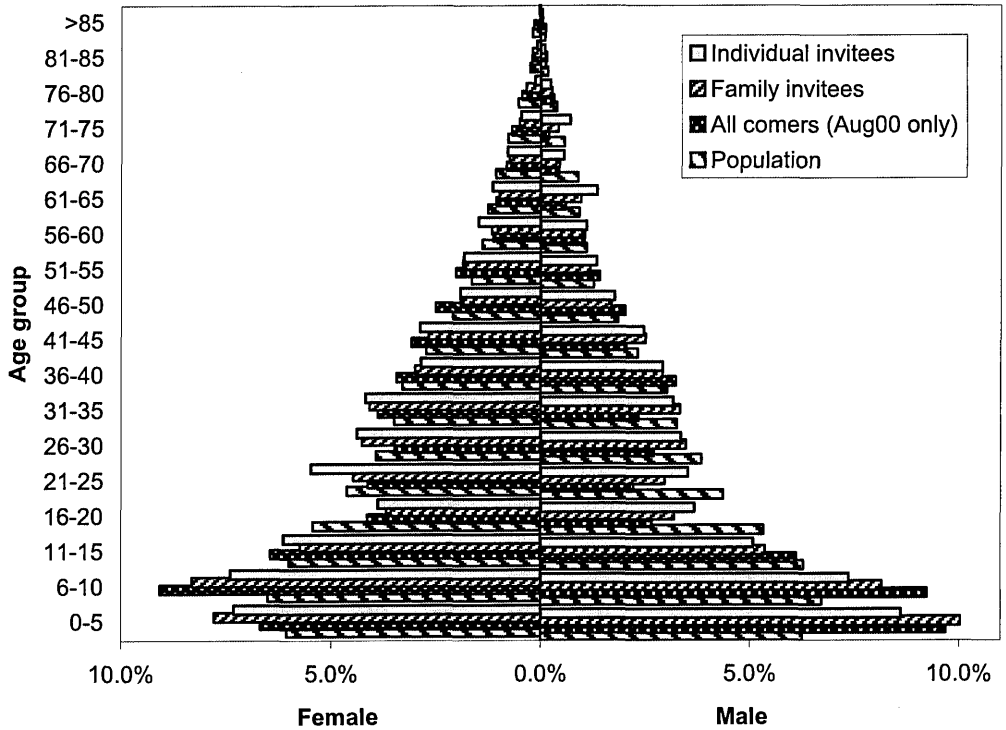


Fig 5.2. Population pyramids by sampling method with population data as comparison.

Children are over represented in the sample, and young adults, particularly men, under represented. This pattern was worst in the unselected survey sample, and progressively improved with inviting families, and then inviting individuals. Calculated population figures have been used for comparison as there were a number of issues with the study

census: some of these were apparent at the outset, such as swathes of families without ages recorded, whilst others became clear as the study progressed, such as gross inconsistencies in ages and number of family members. It is therefore possible that the disparities between sample and population might be due to differences between the age structure in Phước Long and that in the country as a whole (although this is less likely to be the case for the S'tiếng than the Kinh, as Phước Long is home to 30% of the country's S'tiếng, and there is less social discrepancy, and no urban subgroups, amongst the S'tiếng). Fig 5 in appendix 2 shows a comparison between the calculated distribution and the study census data. This suggests that the excess of children, but not the dearth of young adults, might be partly accounted for by such local variation. There was no difference in age and sex distribution between the pre and post December 2000 sampling frames. The remainder of the age related sampling failures probably relate to one of three factors: small children are more likely to be ill, and thus be brought to the survey for medical examination, whilst older children and young adults are more likely to be at school (especially if Kinh) or working in the fields, although one might have expected the latter effect to extend through to middle age. Needlephobia appeared to be commonest in teenagers, which might also account for some invitees staying away.

Age was one of the most powerful predictors of smear positivity in the first survey, so must be considered an important potential confounder of any trends or associations. With the exception of the April 2003 survey, by which time the survey census was sufficiently out of date to exclude most infants and young children, there are no apparent differences in age distribution of surveys (table 5.3 & fig 6, appendix 2). The relative excess of children might lead to an overestimate of population malaria prevalence.

Survey	Mean age	SD
Aug-00	23.1	19.2
Dec-00	23.1	18.7
Mar-01	22.6	18.9
Dec-01	23.7	18.2
Apr-02	24.3	19.0
Aug-02	24.0	19.1
Dec-02	23.9	19.0
Apr-03	25.3	18.3

Table 5.3. Mean age by survey

The S'tiếng appear over represented in all surveys other than August 2000. The hamlets dropped from the sampling frame after December 2000 were disproportionately Kinh, accounting for some of the discrepancy. Official figures for the ethnic composition of the population at hamlet level were not available, but the ethnic breakdown of the hamlets included in all the surveys from the census information is given in the last column of table 5.1. It is clear that the S'tiếng remain over represented, but not to the extent at first apparent. Most of the reasons for this sampling failure have been discussed in Chapter 3, and revolve around the relative poverty of the S'tiếng: a medical examination and a small amount of free medicine proving a much less powerful inducement for the Kinh, many of whom can easily afford health care when necessary. The S'tiếng community health workers appeared more successful at mobilising their communities, possibly due to the different kin structures apparent in different ethnic populations. Ethnicity was such an important predictor of smear positivity that almost all analyses have had to be stratified by or adjusted for ethnic group. Once again, the relative excess of S'tiếng would lead to an overestimate of population malaria prevalence.

Many individuals were included in more than one survey: 1822 in two, 600 in three, 206 in four and 84 in five or more surveys. Repeated observations in these individuals are clearly not independent, although without excluding sampled individuals from future surveys (which would be practicably impossible as well as wrong), some degree of repeated sampling is inevitable. The expected number of repeat observations is much smaller however: assuming the census data to be accurate, and incorporating the household sampling design, 365 individuals would have been expected to figure in two surveys, and only 50 in three. Discarding data from multiply sampled individuals would be a terrible waste of data, but using these individuals robustly is also a challenge.

Parasite prevalence and demographic associations

The prevalence of parasites across all surveys including February 2000 was 10.2%, and the spleen rate in 2 to 9 year olds was 8.6% (6.1% in all ages). These values would classify malaria in the study region as hypoendemic, although endemnicity remains to be established. *P. falciparum* (*Pf*) alone was responsible for over half the infections (52.8%), and was involved in 63% of infections in total, whilst the equivalent figures for *P. vivax* (*Pv*) are 35.6% and 45%. There were 18 infections with *P. malariae* (*Pm*) alone (1.2%), and a further 28 (1.8%) in which this species was involved in mixed infections. In total there were 158 mixed infections (10.8%), of which 82% were *Pf*/*Pv*, 6.5% *Pf*/*Pm*, 2.6% *Pv*/*Pm* and 8.5% *Pf*/*Pv*/*Pm*. The number of mixed infections is greater than expected from the individual species frequencies. Assuming *Pf* and *Pv* infections to be independent, and the relationship between infection and patent parasitaemia (including time course of the latter) to be the same, we would expect 64 mixed *Pf*/*Pv* infections, instead of the 130 we have observed.

Ethnic and geographic heterogeneity

The ethnic differences found in the first survey were mostly consistent throughout subsequent surveys (table 5.4). The one exception was the majority Kinh, who unexpectedly demonstrated a higher prevalence than two of the minority groups in the first survey, but showed the lowest prevalence in the remaining surveys. Once again the possibility of geographical confounding of this association must be explored: the inter-hamlet variation in SPP is displayed in fig 5.3, with depictions of the relationship between the proportion of S'tiếng in the hamlet and hamlet sample in figs 5.4 and 5.5 respectively. The SPP within different ethnic groups within the hamlets are displayed in table 5.6, with the ethnic composition of hamlet samples in table 5.7 for comparison. Once again both geographic and ethnic heterogeneity are apparent, with the S'tiếng suffering a higher burden of malaria and the Kinh lower than all other ethnic groups. Comparing the

inter-hamlet variation between the first and subsequent surveys suggests the differences may not be consistent. Data is only available from Đắc Ơ for all surveys, and whilst the hamlets of Bù Cà, Bù Bưng and Bù Khơn all tend to have higher than average transmission, and those of Đak Lim and Thôn 7 lower than average, the ranking (highest to lowest SPP) has changed considerably, as have the apparent transmission rates in the other hamlets (table 5.5). Whilst some populations, whether ethnically or geographically defined, do have a low prevalence of malaria, these data do not clearly define large sections of the population not at risk from malaria. Lacking permission to map the study area, we cannot investigate geographical microvariations in transmission below the hamlet level.

Ethnicity	SPP (total obs)
Kinh	2.1% (6262)
S'tiếng	14.4% (9004)
Tày	7.6% (410)
Nùng	8.7% (492)
Other	5.9% (135)
Total	9.3% (16303)

Table 5.4. SPP by ethnicity

Hamlet	SPP in 1st survey	Rank	Level	SPP in later surveys	Rank	Level
Thôn 3	18% (126)	5	M	15% (638)	2	H
Thôn 4	20% (284)	4	M-H	5% (613)	6	L
Thôn 6	11% (112)	8	M	9% (533)	5	M
Thôn 7	0 (32)	11	L	5% (216)	7	L
Thôn 9	9% (152)	9	M	4% (601)	9	L
Bù Bưng	30% (220)	2	H	13% (571)	4	M-H
Bù Cà	37% (191)	1	H	13% (640)	3	M-H
Bù Khơn	26% (117)	3	H	25% (505)	1	H
Bù Xia	16% (138)	6	M	4% (312)	10	L
Đak Lim	3% (61)	10	L	1% (117)	11	L
Đak U	13% (189)	7	M	5% (378)	8	L
Total	19.7% (1622)			10.3% (5124)		

Table 5.5. Comparison of SPP in hamlets in Đắc Ơ between first and subsequent surveys. Cell contents are percentage smear positive (total number of observations).

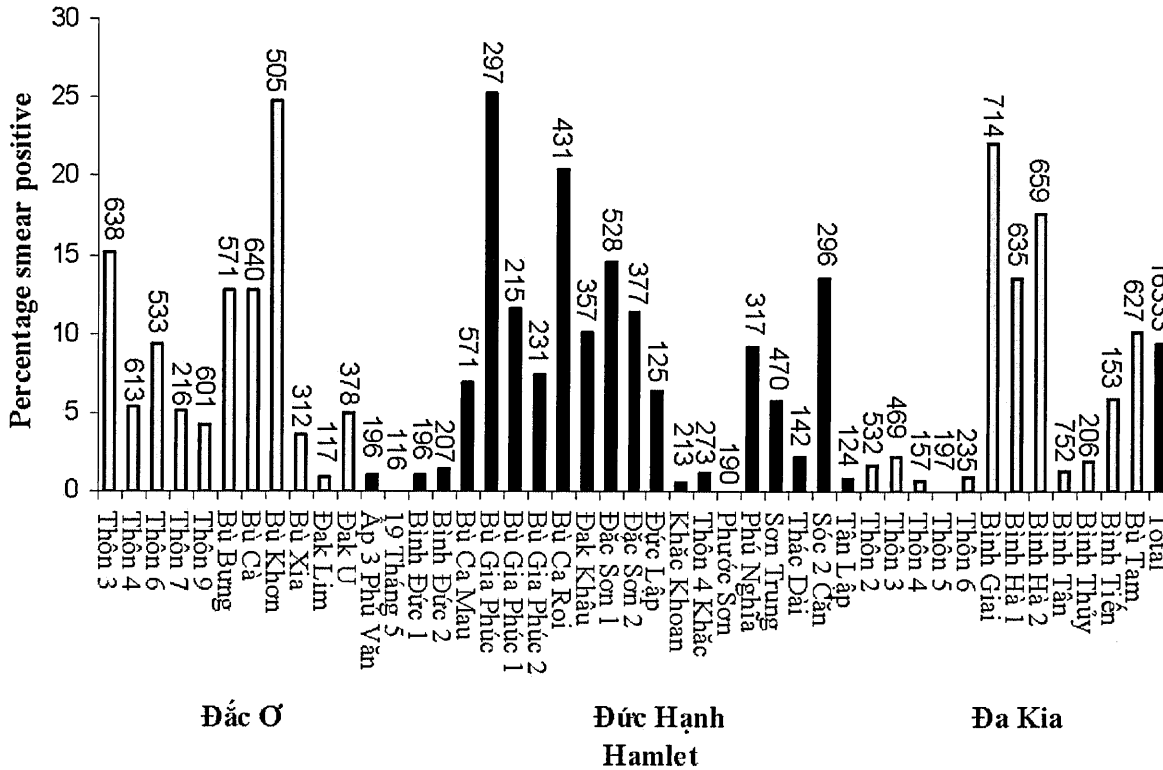


Fig 5.3. Variation in SPP across hamlets. Bar labels are total number of observations.

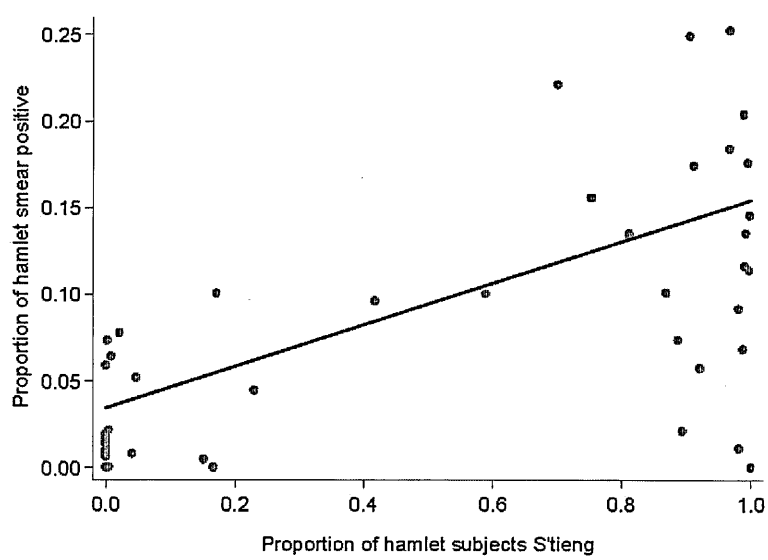


Fig 5.4. Relationship between the proportion of smear positive observations in a hamlet and the proportion of observations in that hamlet on subjects from the S'tiếng ethnic group. Linear regression coefficient 0.11, p<0.001.

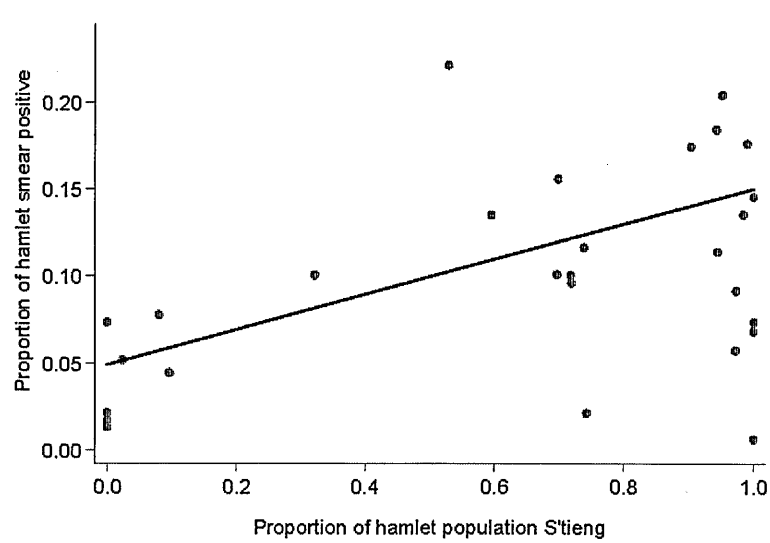


Fig 5.5. Relationship between the proportion of smear positive observations in a hamlet and the proportion of S'tiếng in the hamlet population. Linear regression coefficient 0.08, p=0.009.

Hamlet	Kinh	S'tiếng	Tày	Nùng
Thôn 3	4% (85)	**18% (497)**	12% (43)	0 (13)
Thôn 4	3% (31)	5% (340)	5% (107)	**7% (123)**
Thôn 6	2% (248)	**17% (252)**	7% (14)	
Thôn 7	2% (129)	8% (52)	**13% (30)**	
Thôn 9	4% (563)	**12% (25)**		
Bù Bưng	3% (38)	**14% (530)**		
Bù Cà	0 (20)	**13% (617)**		
Bù Khơn	0 (37)	**27% (463)**		
Bù Xia	4% (293)			
Đak Lim	1% (112)			
Đak U	5% (362)			
Ấp 3 Phú Văn	1% (193)			
19 Tháng 5	0 (116)			
Bình Đức 1	1% (191)			
Bình Đức 2	2% (204)			
Bù Ca Mau		7% (564)		
Bù Gia Phúc	11% (9)	**26% (287)**		
Bù Gia Phúc 1		12% (213)		
Bù Gia Phúc 2	4% (25)	**8% (205)**		
Bù Ca Roi		21% (426)		
Đak Khâu	2% (46)	**11% (310)**		
Đắc Sơn 1		15% (527)		
Đắc Sơn 2		11% (376)		
Đức Lập	7% (122)			
Khăc Khoan	**1% (174)**	0 (32)		
Thôn 4 Khăc Khoan		1% (268)		
Phước Sơn	0 (189)			
Phú Nghĩa		9% (311)		
Sóc 2 Căn	13% (46)	13% (240)		
Sơn Trung	0 (35)	**6% (433)**		
Thác Dài	0 (13)	**2% (127)**		
Tân Lập	0 (118)			
Thôn 2 ĐK	2% (527)			
Thôn 3 ĐK	2% (462)			
Thôn 4 ĐK	0 (143)			
Thôn 5 ĐK	0 (191)			
Thôn 6 ĐK	1% (233)			
Bình Giải	2% (195)	**31% (500)**	0 (15)	
Bình Hà 1	20% (5)	**14% (630)**		
Bình Hà 2		18% (656)		
Bình Tân	1% (737)			
Bình Thủy	2% (204)			
Bình Tiến	**5% (118)**			0 (15)
Bù Tam	0 (21)	**13% (107)**	8% (173)	11% (321)

Table 5.6. SPP amongst different ethnic groups by hamlet. Only those ethnic groups with more than 10 observations in a hamlet sample are included. Cell contents SPP (total number of observations). Highest SPP in a hamlet is in bold for those hamlets with 2 or more ethnic groups with sufficient representatives in the surveys.

Commune and hamlet		Ethnic Group					Total number
		Kinh	S'tiếng	Tày	Nùng	Other	
ĐẮC Ơ	Thôn 3	13%	78%	7%	2%	0	638
	Thôn 4	5%	56%	18%	20%	2%	611
	Thôn 6	47%	47%	3%	1%	3%	532
	Thôn 7	60%	24%	14%	2%	1%	216
	Thôn 9	94%	4%	1%	0	1%	598
	Bù Bưng	7%	93%	0	0	1%	571
	Bù Cà	3%	96%	0	0	1%	640
	Bù Khơn	7%	92%	0	0	1%	505
	Bù Xia	96%	0	<1%	<1%	3%	304
	Đak Lim	96%	0	0	4%	0%	117
	Đak U	96%	1%	0	0	3%	378
ĐỨC HẠNH	Ấp 3 Phú Văn	100%	0	0	0	0	193
	19 Tháng 5	100%	0	0	0	0	116
	Bình Đức 1	100%	0	0	0	0	191
	Bình Đức 2	100%	0	0	0	0	204
	Bù Ca Mau	0	99%	0	0	1%	571
	Bù Gia Phúc	3%	97%	0	0	<1%	297
	Bù Gia Phúc 1	1%	99%	0	0	0	215
	Bù Gia Phúc 2	11%	89%	0	0	<1%	231
	Bù Ca Roi	1%	99%	0	0	0	431
	Đak Khâu	13%	87%	0	0	<1%	357
	Đắc Sơn 1	<1%	>99%	0	0	0	528
	Đắc Sơn 2	<1%	>99%	0	0	0	377
	Đức Lập	99%	1%	0	0	0	123
	Khăc Khoan	83%	15%	2%	0	0	211
	Thôn 4 Khăc Khoan	2%	98%	0	0	0	273
	Phước Sơn	100%	0	0	0	0	189
	Phú Nghĩa	1%	98%	0	0	1%	317
	Sóc 2 Căn	16%	81%	0	0	3%	296
	Sơn Trung	8%	92%	0	0	<1%	470
	Thác Đài	9%	89%	0	0	1%	142
	Tân Lập	96%	4%	0	0	<1%	123
ĐA KIA	Thôn 2 ĐK	99%	0	0	0	1%	530
	Thôn 3 ĐK	99%	<1%	<1%	0	1%	469
	Thôn 4 ĐK	92%	0	3%	3%	2%	155
	Thôn 5 ĐK	97%	1%	0	0	3%	197
	Thôn 6 ĐK	99%	0	0	0	1%	235
	Bình Giải	27%	70%	2%	<1%	<1%	714
	Bình Hà 1	1%	99%	0	0	0	635
	Bình Hà 2	<1%	>99%	0	0	0	659
	Bình Tân	98%	0	1%	0	1%	751
	Bình Thủy	99%	0	1%	0	1%	206
	Bình Tiến	79%	0	4%	10	7%	150
	Bù Tam	3%	17%	28%	51%	1%	626
Total		38.4%	55.3%	2.5%	3%	0.8%	16293

Table 5.7. Variation in ethnic composition of sample by hamlet.

Species	Kinh	S'tiêng	Tày	Nùng	Other	Number
Pf	46%	53%	58%	56%	50%	794
Pv	50%	34%	36%	37%	50%	536
Pm	0%	1%	0	0	0	18
Pf/Pv	4%	9%	7%	7%	0	130
Pf/Pm	1%	1%	0	0	0	11
Pv/Pm	0	<1%	0	0	0	4
Pf/Pv/Pm	0	1%	0	0	0	13
Total smear positive	133	1,291	31	43	8	1,506

Table 5.8. Species variation by ethnic group.

The suspect association between *P. vivax* and the Nùng ethnic group was not borne out in subsequent surveys, although the Kinh do seem to have a higher proportion of vivax (table 5.8). The potential confounders of this association are age, location and season. There were minimal differences in age distribution between ethnic groups (table 5.9), the Kinh sample actually having slightly fewer children (at higher risk of *P. vivax* infection – see below). Disentangling the effects of geography and season becomes increasingly difficult as the number of observations in any category shrinks. There are several hamlets in which a *P. vivax* infection is the only positive smear amongst all Kinh subjects in that hamlet (table 13, appendix 2). In the absence of routine radical curative therapy, many of these may be relapses. Restricting our definition of smear positivity to falciparum in light of this possibility reveals a number of hamlets without apparent transmission (table 5.10). Most of these hamlets were dropped from the sampling frame after the December 2000 survey, however, thus were they to be transmission free, they would still not comprise a sufficiently large malaria free population to account for the dearth of mixed infections.

Age group	Kinh	S'tiêng	Tày	Nùng	Other	Total
<1	1.9%	1.9%	0.5%	0.2%	2.2%	1.8%
1	2.4%	2.5%	1.7%	4.1%	7.4%	2.5%
2-4	9.9%	10.4%	9.3%	11.4%	13.3%	10.3%
5-9	15.4%	17.0%	16.8%	15.7%	16.3%	16.3%
10-14	13.5%	11.5%	9.3%	8.1%	8.2%	12.1%
15-19	7.3%	7.9%	5.4%	9.4%	3.7%	7.6%
20-29	14.3%	15.8%	21.0%	19.9%	14.1%	15.4%
30-39	15.1%	12.0%	16.8%	13.4%	16.3%	13.4%
40-49	10.8%	8.1%	9.8%	7.1%	6.7%	9.1%
50-59	4.9%	6.2%	5.4%	6.7%	5.9%	5.7%
60-69	2.7%	4.3%	3.2%	2.9%	2.2%	3.6%
70+	2.0%	2.4%	1.0%	1.2%	3.7%	2.2%
Mean Age	23.4	23.5	24.2	22.8	23.1	23.5

Table 5.9. Age distribution of ethnic subsamples.

Hamlet	Kinh	S'tiếng	Tày	Nùng
Thôn 3	1% (85)	12% (497)	9% (43)	0 (13)
Thôn 4	0 (31)	3% (340)	4% (107)	2% (123)
Thôn 6	<1% (248)	10% (252)	0 (14)	
Thôn 7	0 (129)	4% (52)	3% (30)	
Thôn 9	2% (563)	12% (25)		
Bù Bưng	0 (38)	8% (530)		
Bù Cà	0 (20)	9% (617)		
Bù Khơn	0 (37)	18% (463)		
Bù Xia	2% (293)			
Đak Lim	0 (112)			
Đak U	2% (362)			
Ấp 3 Phú Văn	0 (193)			
19 Tháng 5	0 (116)			
Bình Đức 1	1% (191)			
Bình Đức 2	2% (204)			
Bù Ca Mau		4% (564)		
Bù Gia Phúc		15% (287)		
Bù Gia Phúc 1		5% (213)		
Bù Gia Phúc 2	0 (25)	4% (205)		
Bù Ca Roi		11% (426)		
Đak Khâu	2% (46)	7% (310)		
Đắc Sơn 1		8% (527)		
Đắc Sơn 2		7% (376)		
Đức Lập	3% (122)			
Khắc Khoan	0 (174)	0 (32)		
Thôn 4 Khắc Khoan		1% (268)		
Phước Sơn	0 (189)			
Phú Nghĩa		7% (311)		
Sóc 2 Căn	11% (46)	10% (240)		
Sơn Trung	0 (35)	4% (433)		
Thác Dài	0 (13)	0 (127)		
Tân Lập	0 (118)			
Thôn 2 ĐK	1% (527)			
Thôn 3 ĐK	1% (462)			
Thôn 4 ĐK	0 (143)		25% (4)	
Thôn 5 ĐK	0 (191)			
Thôn 6 ĐK	0 (233)			
Bình Giải	2% (195)	23% (500)	0 (15)	
Bình Hà 1		9% (630)		
Bình Hà 2		11% (656)		
Bình Tân	1% (737)			
Bình Thuỷ	1% (204)			
Bình Tiến	4% (118)			0 (15)
Bù Tam	0 (21)	12% (107)	6% (173)	8% (321)
Total	1.1% (6254)	9.2% (9002)	4.9% (410)	5.5% (492)

Table 5.10. Percentage of *P. falciparum* positive observations by hamlet and ethnic group (total observations in hamlet-ethnic group subsample). Shaded hamlets are those dropped from sampling frame after the December 2000 survey. Blank cells denote no observations in that hamlet/ethnic group category

Age, sex and pregnancy

The relationship between age and smear positivity is depicted in fig 5.6. The pattern is similar to that seen in the first survey, as is the ethnic variation in this relationship (fig 5.7), although the Kinh curve is much flatter, in keeping with the generally lower prevalence, and the Tày and Nùng curves have more shape, in keeping with the greater prevalence in the former and the larger number of observations in the latter. Smear positive S'tiếng were younger than smear positive Kinh (mean age 17.1 vs 25.7, $p<0.001$). The Tày (mean age 28.5) and Nùng (28.3) smear positive subjects were not significantly older than the Kinh.

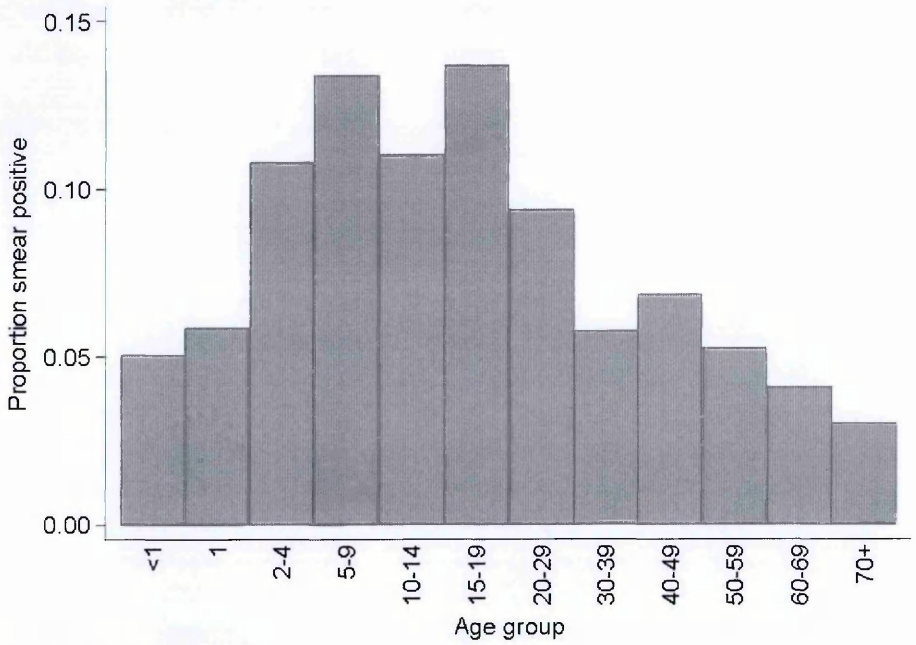


Fig 5.6. Age and SPP

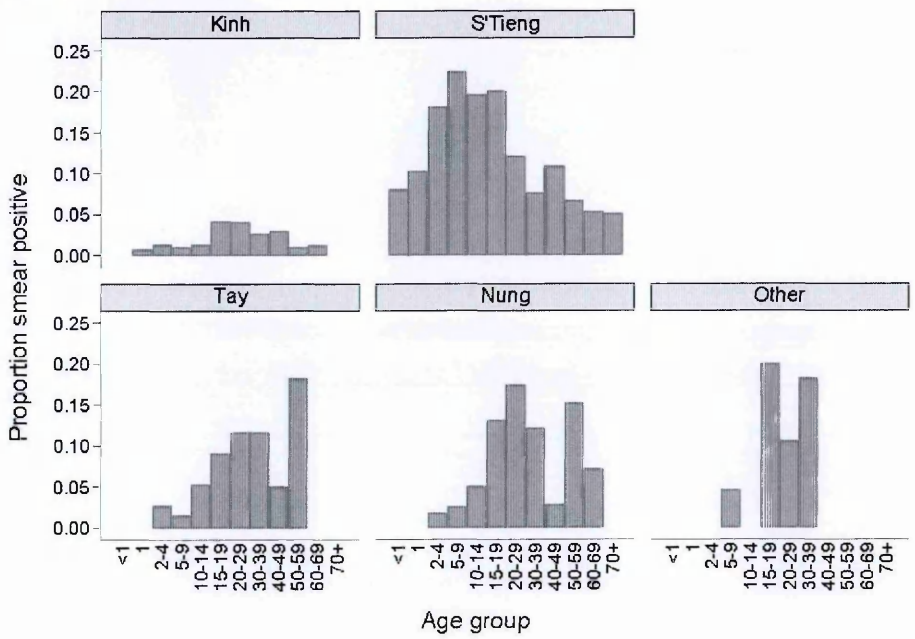


Fig 5.7. Age and SPP by ethnic group.

P. vivax was more common in children (52% of smear positive under 10's compared to 41% of those 10 or over, p<0.001). As in the first survey, this is related to the higher proportion of mixed infections in these age groups (table 5.11), although whether this represents cause or effect is impossible to tell.

Species Age group	*Pf*	*Pv*	*Pm*	*Pf/Pv*	*Pf/Pm*	*Pv/Pm*	*Pf/Pv/Pm*	All *Pv*	All *Pf*	Total smear positive
<1	60%	27%	0	13%	0	0	0	40%	73%	15
1	38%	58%	0	4%	0	0	0	63%	42%	24
2-4	45%	41%	1%	12%	1%	0	1%	54%	58%	180
5-9	47%	38%	1%	12%	1%	<1%	2%	51%	61%	354
10-14	55%	31%	2%	8%	2%	0	1%	41%	66%	217
15-19	59%	31%	2%	7%	0	1%	0	39%	66%	169
20-29	57%	34%	2%	5%	<1%	1%	1%	41%	63%	236
30-39	55%	39%	1%	5%	0	0	1%	44%	61%	127
40-49	57%	32%	1%	9%	1%	0	0	41%	67%	103
50-59	57%	37%	0	6%	0	0	0	43%	63%	49
60-69	58%	25%	4%	13%	0	0	0	38%	71%	24
70+	64%	36%	0	0	0	0	0	36%	64%	11
Total	796	537	18	130	11	4	13	(684)	(950)	1509

Table 5.11. Age and species. Cell contents are percentage of subjects in specified age group positive with specified species or combination of species.

There was less malaria amongst women than men, although the differences were small (table 5.12). This effect was consistent in magnitude and direction across surveys and ethnic groups, with more marked differences in Tày and Nùng (table 5.12). The data from these surveys confirms that the sex difference is confined to adults (table 5.13).

Ethnicity	Male	Female	Total	Number	p
Kinh	2.8%	1.5%	2.1%	6257	<0.001
S'tiêng	15.7%	13.2%	14.4%	9004	0.001
Tày	10.9%	3.9%	7.3%	409	0.006
Nùng	12.5%	4.7%	8.8%	491	0.002
Other	5.8%	6.1%	5.9%	135	0.948
Total	10.4%	8.2%	9.3%	16296	<0.001

Table 5.12. Percentage smear positive by sex and ethnic group.
Number=total number of observations in that ethnic group.

Pregnancy was a significant risk factor for smear positivity: 6.9% of 2168 non-pregnant women harboured parasites compared to 11.6% of 233 pregnant women (p=0.009),

although 1418 observations in women of child bearing age had no pregnancy status recorded (of which 5.4% were smear positive), weakening the validity of this finding.

Kinh				
Age group	Male	Female	Total	Number
<1	0	0	0	115
1	0	1%	1%	148
2-4	1%	2%	1%	619
5-9	1%	1%	1%	961
10-14	2%	1%	1%	847
15-19	7%	2%	4%	456
20-29	7%	2%	4%	892
30-39	4%	2%	3%	946
40-49	4%	3%	3%	674
50-59	1%	1%	1%	305
60-69	3%	0	1%	168
70+	0	0	0	126
Total	2.8%	1.5%	2.1%	6,257

S'tiêng				
Age group	Male	Female	Total	Number
<1	5%	11%	8%	175
1	11%	9%	10%	224
2-4	18%	18%	18%	940
5-9	23%	22%	22%	1,528
10-14	21%	18%	20%	1,031
15-19	21%	19%	20%	709
20-29	16%	10%	12%	1,418
30-39	9%	7%	8%	1,083
40-49	12%	10%	11%	732
50-59	9%	5%	7%	557
60-69	5%	6%	5%	390
70+	7%	3%	5%	217
Total	15.7%	13.2%	14.4%	9,004

Tày				
Age group	Male	Female	Total	Number
<1	0		0	1
1	0	0	0	7
2-4	5%	0	3%	38
5-9	3%	0	1%	69
10-14	7%	4%	5%	38
15-19	8%	11%	9%	22
20-29	27%	2%	12%	86
30-39	16%	4%	12%	69
40-49	10%	0	5%	40
50-59	0	40%	18%	22
60-69	0	0	0	13
70+	0	0	0	4
Total	10.9%	3.9%	7.3%	409

Nùng				
Age group	Male	Female	Total	Number
<1	0	0	0	1
1	0	0	0	20
2-4	0	4%	2%	56
5-9	3%	2%	3%	77
10-14	10%	0	5%	40
15-19	21%	5%	13%	46
20-29	24%	8%	17%	98
30-39	17%	8%	12%	66
40-49	7%	0	3%	35
50-59	19%	12%	15%	33
60-69	11%	0	7%	14
70+	0	0	0	5
Total	12.5%	4.7%	8.8%	491

Table 5.13. Ethnic comparison of the relationship between age, sex & smear positivity. Cell contents are percentage smear positive, or total number of observations in that ethnic and age group category in "Number" column.

Parasitaemia

Only those slides demonstrating pure *P. falciparum* infections were counted. Parasite counts per 400 white blood cells or 1000 red blood cells were converted to parasites per microlitre by assuming an average WBC count of $8x10^9$/l and an average RBC count of $4x10^{12}$/l. Parasite counts were then log transformed for further analysis. There was no

difference in parasitaemia between hamlets or ethnic groups (fig 5.8). There was a significant negative correlation with age (fig 5.9), which varied by ethnic group (fig 5.10)

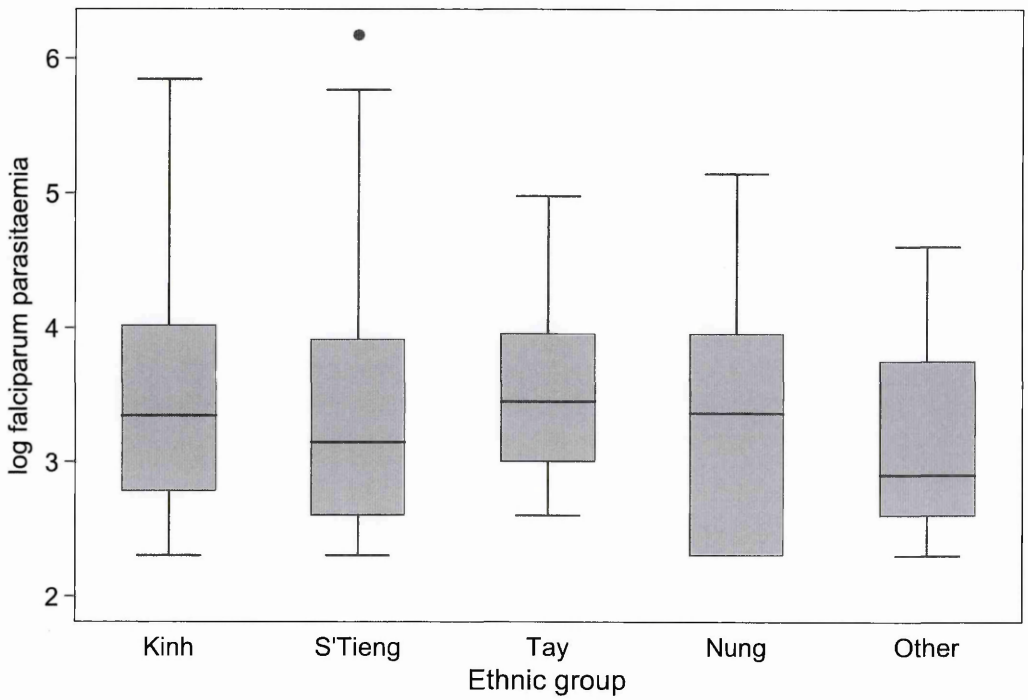


Fig 5.8. Variation of *P. falciparum* parasitaemia with ethnic group.

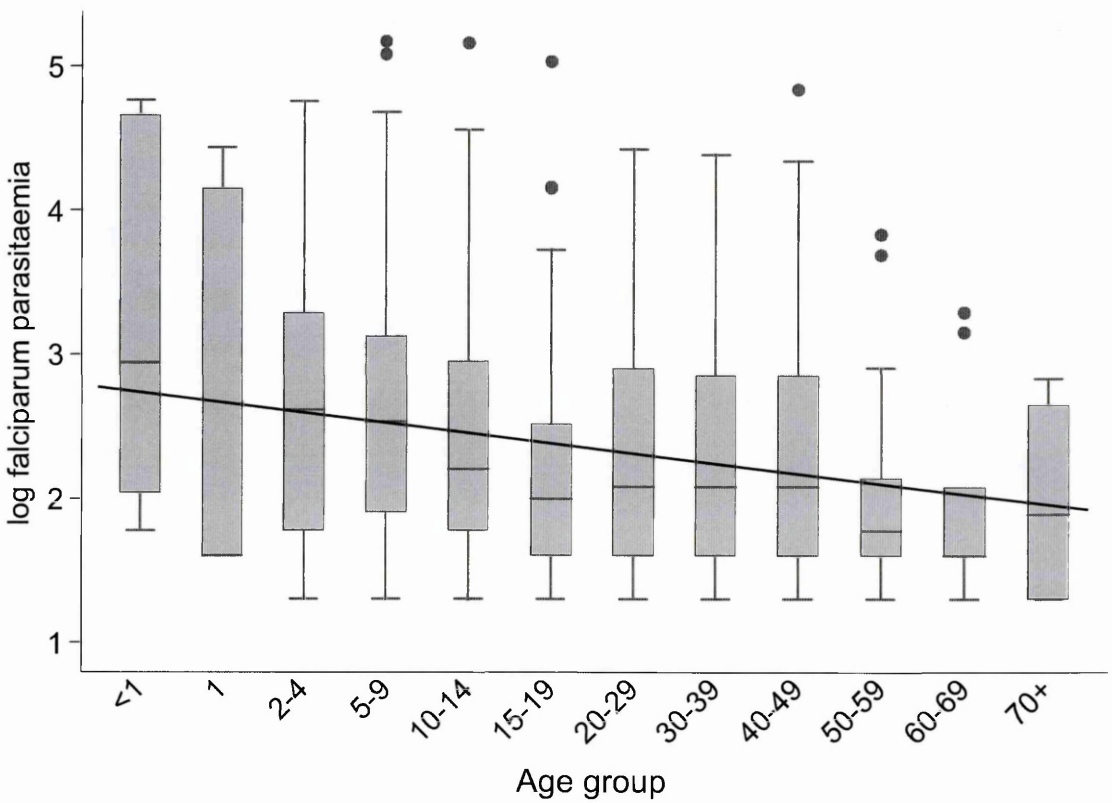


Fig 5.9. Variation of *P. falciparum* parasitaemia with age group. Linear regression coefficient of log *P. falciparum parasitaemia* on age=-0.01, p<0.001.

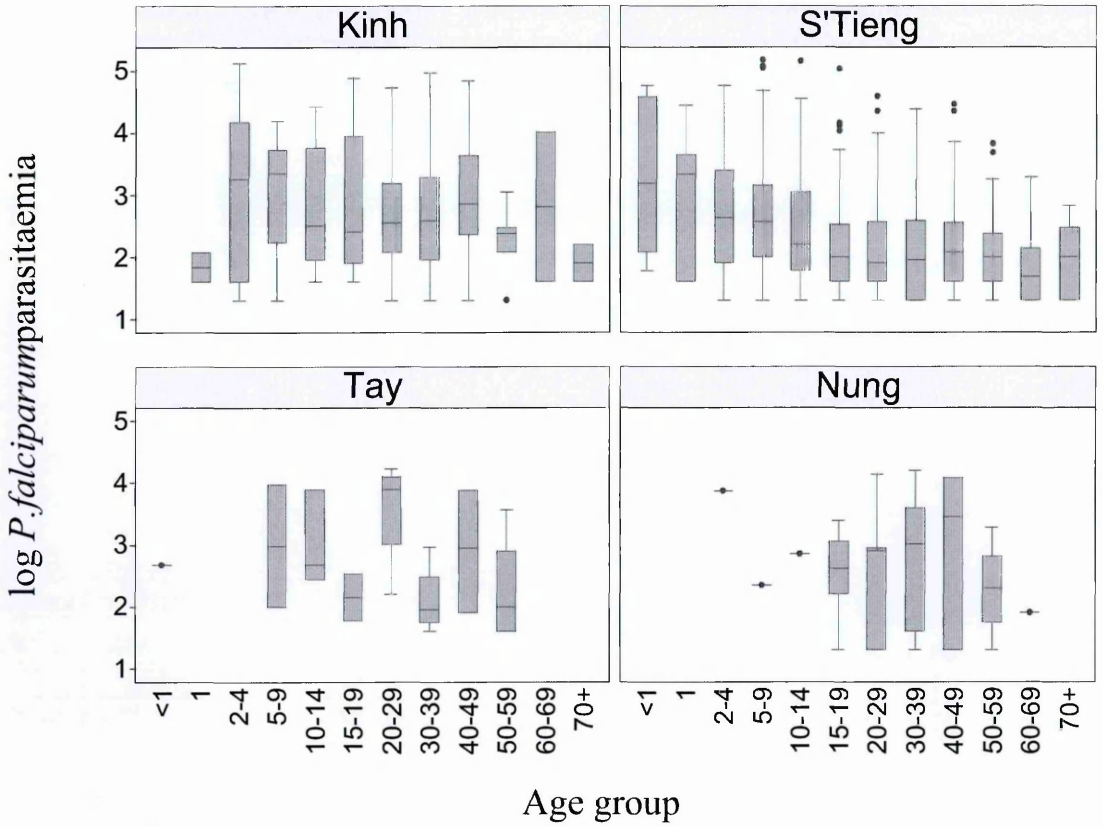


Fig 5.10. Differences in relationship between *P. falciparum* parasitaemia and age group between ethnic groups. Linear regression coefficients of *P. falciparum* parasitaemia on age: Kinh +0.004, p=0.68; S'tiếng –0.13, p<0.001; Tày +0.0002, p=0.99; Nùng –0.02, p=0.03.

Gametocyte carriage

Gametocytes were observed in 2.4% of the slides (25% of the positive slides). Subjects harbouring *P. falciparum* were more likely to carry gametocytes than were those carrying *P. vivax* (35% vs 6% p<0.001). *P. falciparum* gametocytes were found more often in mixed infections than isolated *Pf* infections (56% vs 32% p<0.001). There was a trend towards increasing gametocyte carriage (as a proportion of those who were smear positive) with increasing transmission, but the relationship was inconsistent (fig 5.11). There was no sex difference in gametocyte carriage (2.2% of observations in females and 48.3% of smear positive observations in females vs 2.5% and 51.7% of male equivalents), but a trend towards decreasing carriage with increasing age was apparent (table 5.14).

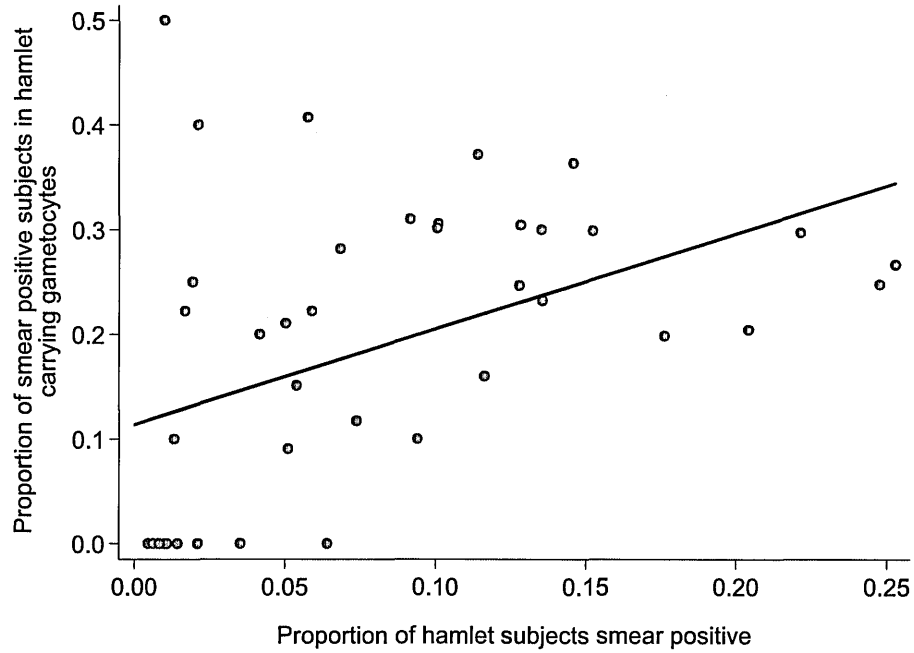


Fig 5.11. Proportion of smear positive subjects carrying gametocytes vs smear positive prevalence by hamlet, with regression line fitted. Linear regression coefficient 0.64, p=0.01

Age group	Proportion of population carrying gametocytes	Proportion of smear positive subjects carrying gametocytes	Number of observations	Number of smear positive obs
<1	3.0%	60%	297	15
1	1.5%	25%	409	24
2-4	3.4%	31%	1674	181
5-9	3.8%	28%	2660	356
10-14	2.9%	26%	1969	217
15-19	2.7%	20%	1240	170
20-29	1.9%	20%	2518	237
30-39	1.3%	23%	2195	127
40-49	1.8%	26%	1499	103
50-59	1.4%	27%	931	49
60-69	0.5%	13%	589	24
70+	0.8%	27%	365	11
Total	2.4%	25.4%	16346	1514

Table 5.14. Gametocyte carriage and age. Under 10 vs over 10 p=0.001.

Temporal trends

Parasite prevalence

The smear positive prevalence in each survey is displayed alongside monthly precipitation data in fig 5.12, with additional depiction of relative frequencies of *P. vivax* and *P. falciparum*. A negative secular trend is readily apparent, and there is little seasonal variation. Despite the secular decline, it is clear from the survey data that malaria is

endemic in the study region. The average spleen and smear positive prevalences over the course of the surveys would classify transmission as hypoendemic. Individual survey spleen rates in 2-9 year olds and all age groups, together with the SPP's for comparison, are shown in table 5.15 and rendered graphically in fig 5.13. Whilst the smear data would classify the study area as mesoendemic during the earlier years of the study, the spleen rates are consistently hypoendemic.

P. falciparum is the dominant parasite throughout the year, although the relationship between *P. vivax* and *falciparum* does appear to show some seasonal variation.

A secular decline in rainfall over the period under study is also apparent: according to the weather station in the centre of Phước Long district, 3328mm of rain fell in 2000, 2891mm in 2001, 2614mm in 2002 and 2429mm in 2003.

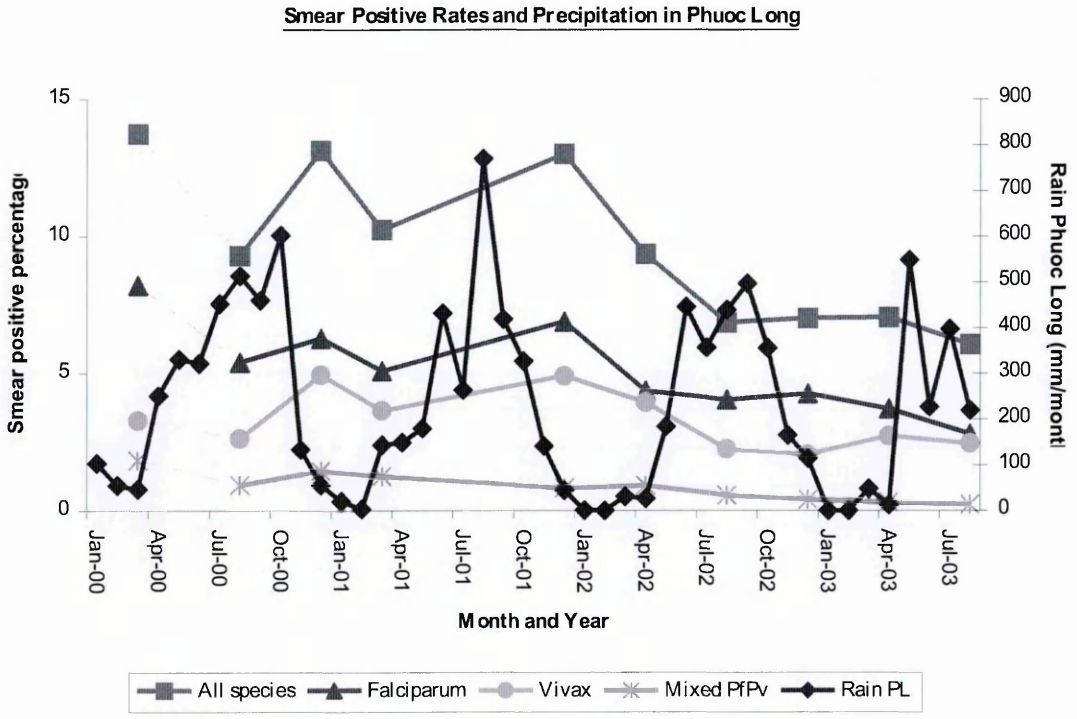


Fig 5.12. Precipitation and smear positive prevalence across all surveys (Feb 2000 data shown but separated by dotted line as survey conducted in different location).

	Aug 00	Dec 00	Mar 01	Dec 01	Apr 02	Aug 02	Dec 02	Apr 03	Total
Spleen rate 2-9 years	5.8%	6.5%	13.2%	5.9%	3.2%	7.3%	3.9%	2.0%	6.4%
SPP 2-9 year old's	10.8%	16.7%	17.7%	10.0%	7.9%	9.5%	8.7%	5.0%	12.4%
Spleen rate all ages	3.8%	4.9%	10.2%	6.7%	2.5%	2.9%	4.3%	2.7%	4.9%
SPP all ages	9.2%	11.9%	12.7%	8.8%	5.5%	6.8%	6.6%	6.1%	9.3%

Table 5.15. Spleen rates and smear positive prevalence in 2 to 9 year olds and all ages.

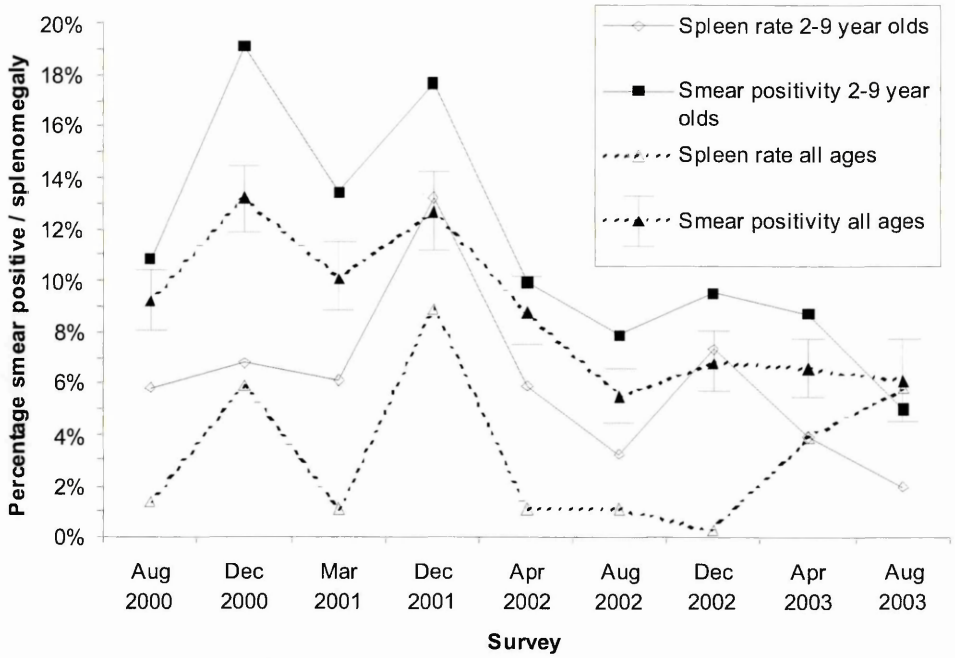


Fig 5.13. Spleen and smear positive prevalence for 2 to 9 year olds and all ages. Error bars on overall smear positivity are 95% confidence intervals for estimate of SPP. p value for regression of smear positivity on survey <0.001.

Decline in malaria prevalence

The variation in survey sampling methods over time raised the possibility that this decline was artefactual. The four significant changes in sampling methods were the move from an open invitation to inviting families in December 2000, the dropping of certain hamlets from the sampling frame after December 2000, the move from inviting families to inviting individuals in April 2002, and the central construction of a list of invitees in August 2003. The first of these changes might have been expected to decrease the SPP, whereas an increase is apparent. This is likely to be due to the proportion of S'tiếng present in the December 2000 survey being higher than that in the August survey (table 5.2). The change in sampling frame after December 2000 would be expected to increase the crude smear positive prevalence. This effect is seen, although may have been contributed to by the greater excess of S'tiếng seen in the March 2001 survey than any other. The SPP in the hamlets included in all the surveys subsequent to December 2000 was 13.6% in the August 2000 survey (compared to 9.2% across all hamlets in that survey), and 16.4% in the December 2000 survey (compared to 11.9%). There was little discernable effect of the

family sampling on the age structure of the sample (fig 5.2), and the proportion of high risk age groups was consistent throughout, as was the sex ratio (table 5.16). The family sampling strategy should not bias the estimate of SPP: if malaria clusters in families, then the extra members from "malarious" families should be balanced by those from "non-malarious" families. If "malarious" families tend to be larger, then the members of those families would have an increased chance of being sampled by a random individual sampling strategy. If the sampling strategy is not random, any systematic bias is likely to operate in a similar direction in both strategies: if the local health workers tended to encourage ill members of the community to attend, the family sampling strategy might be expected to dilute this effect (with well family members from a household with unwell individuals) and thus result in a lower estimate of SPP. The properly random sampling of the August 2003 survey might be expected to result in a lower, less biased estimate of the SPP. The decline in prevalence is clear well before this survey, however, which also has a number of other differences from previous surveys in that the hamlets included in the simultaneous KAP study were excluded.

	Age group						Proportion	Total
Survey	<2	2-9	10-19	20-39	40-59	60+	male	number
Aug-00	4.7%	27.4%	21.5%	25.3%	15.5%	5.6%	46.5%	2469
Dec-00	4.5%	28.0%	19.0%	28.2%	15.0%	5.3%	48.5%	4622
Mar-01	6.0%	27.7%	18.3%	29.2%	13.0%	5.8%	48.3%	1840
Apr-02	3.8%	23.7%	21.4%	31.9%	14.1%	5.3%	50.4%	1573
Aug-02	3.3%	25.4%	19.8%	30.4%	14.7%	6.5%	48.7%	1707
Dec-02	5.1%	24.7%	19.2%	29.2%	15.2%	6.6%	46.8%	1660
Apr-03	4.2%	27.4%	17.2%	29.7%	15.9%	5.7%	47.5%	1602
Aug-03	0.8%	23.5%	22.8%	31.1%	16.1%	5.6%	49.2%	851
Total	4.3%	26.6%	19.7%	28.9%	14.9%	5.7%	48.2%	16324

Table 5.16. Age group and sex distribution of samples by survey. Cell contents are percentage of survey subjects except total number of subjects column.

In addition to the changes in survey technique, differences in the proportion of sample drawn from high risk areas or age or ethnic groups should be considered as confounders. Only two such differences are apparent: the proportion of S'tiêng subjects increases between the August 2000 and March 2001 surveys, but then remains stable (table 5.2), and

a higher mean age of the April 03 subjects compared to previous surveys (table 5.3). Neither of these has an important impact on the overall trend.

The reported incidence of malaria at local health stations also declined over this period (detailed data is unfortunately not available), as did the admissions for severe malaria (see Chapter 7), providing corroboration for the survey data.

The decline in prevalence was not seen in all ethnic groups (fig 5.14a & b). It was most prominent amongst the S'tiếng, but also seen in the Nùng (although this was not statistically significant, given the small number of observations amongst the Nùng). Prevalence amongst the Tày and Kinh appear essentially unchanged over time (the apparent rise in the April 2003 survey being an artefact of small sample size – the Tày point representing 1 smear positive subject out of 7, and the Nùng 1 out of 11).

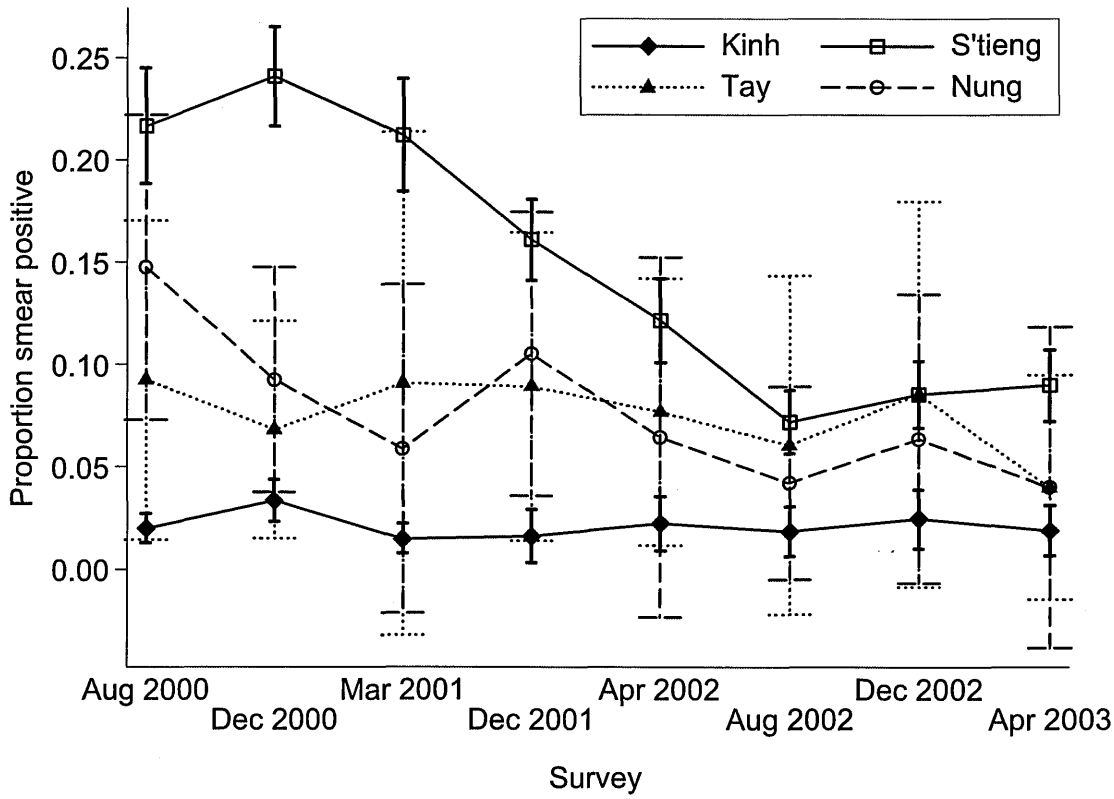


Fig 5.14a. Trends in smear positive prevalence over time by ethnic group. Error bars represent 95% confidence intervals. Aug 2003 survey not shown as Tày and Nùng 95% CI distort scale (-0.21 – 0.49 and –0.10 – 0.27 respectively).

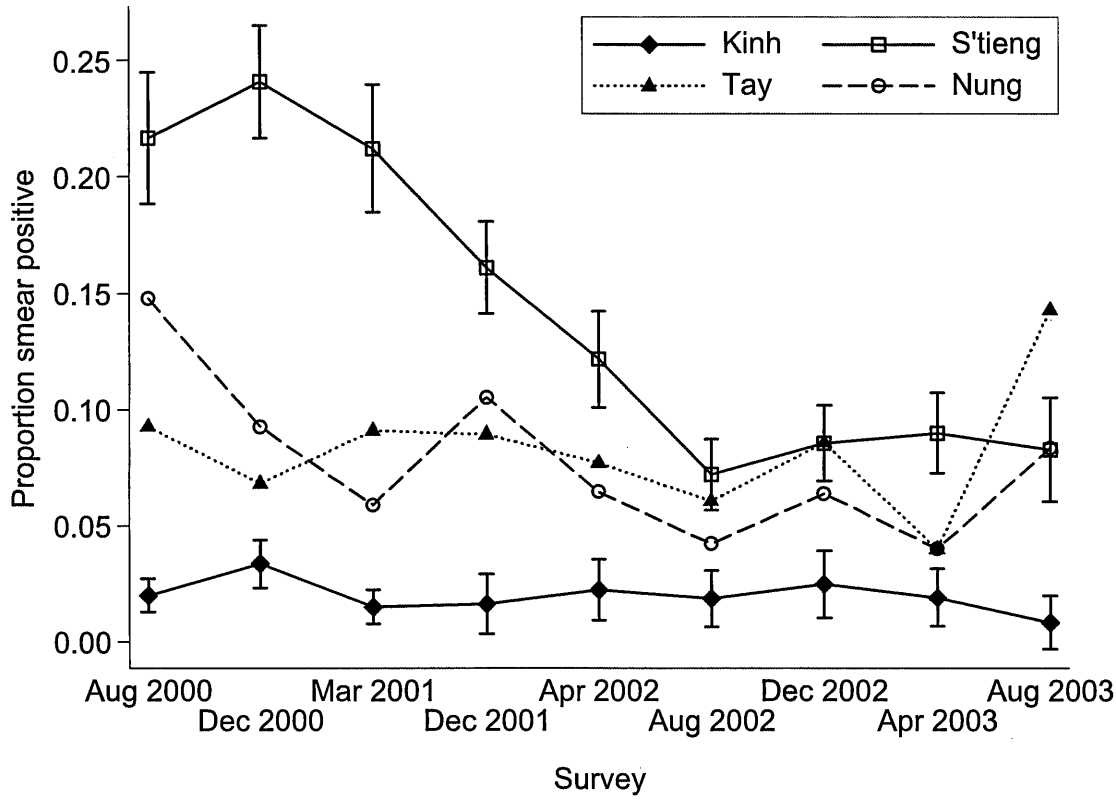


Fig 5.14b. Trends in smear positive prevalence over time by ethnic group. Error bars as for fig 5.14a, but shown for Kinh and S'tiếng only.

Symptomatology

Fever, both reported and measured, headache, rigors and chills had been adopted as indicators of symptomatic malaria being symptomatic on the basis of published data. One or more were present in 47% of smear positive episodes, of which 18% involved headache alone. Survey subjects also reported symptoms in 32% of smear negative episodes, of which a third were headache alone. A slightly higher proportion of subjects with *P. falciparum* reported symptoms than those carrying *P. vivax* (49 vs 44%, p=0.06). The proportion of subjects with mixed infections exhibiting symptoms was consistent with that for falciparum.

The proportion of parasitaemic subjects with symptoms did not decrease with age, contrary to expectation. Smear negative individuals appeared to report more symptoms during early adulthood and middle age, which was again unexpected, as non-malarial febrile illnesses tend to be more prevalent in the young. Once subjects reporting headache alone were

excluded, however, a decrease in symptomatology with age was apparent, although perhaps not as steep as expected (fig 5.16). Only a small fraction of the survey subjects were pyrexial at the time of the survey, and the risk of documented fever accompanying a positive blood film decreased sharply with age (fig 5.16).

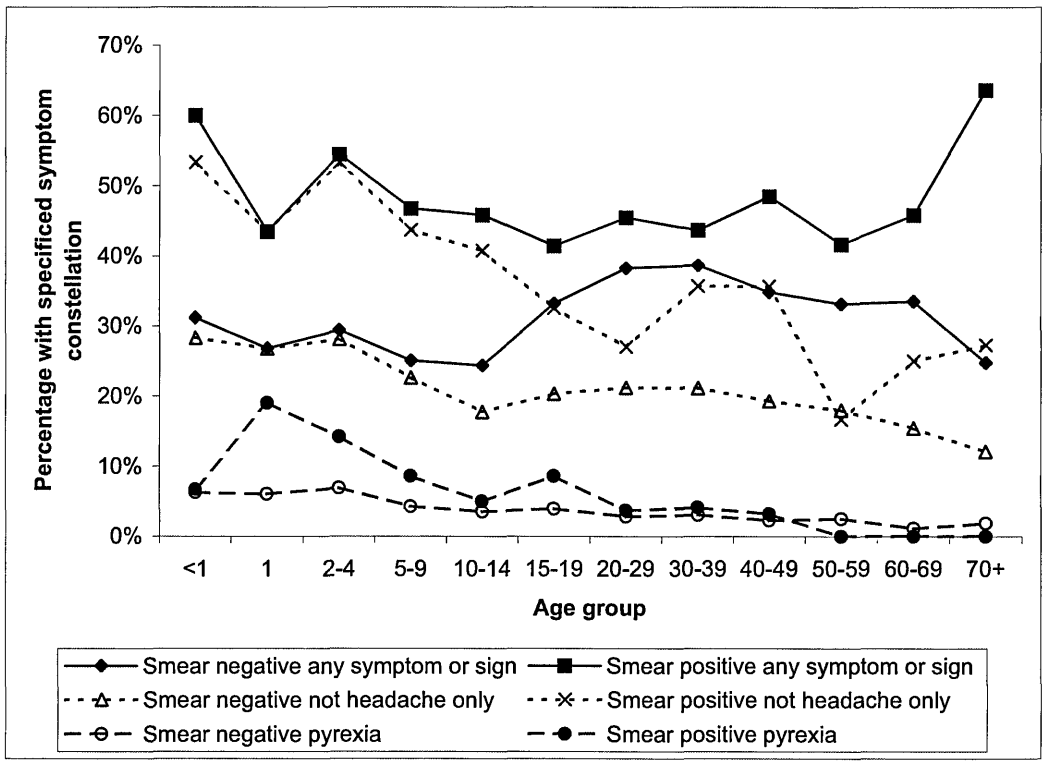


Fig 5.16. Symptomatology and age. Significant negative associations between age and pyrexia (with and without parasitaemia), and reporting symptoms or displaying signs excluding headache alone (with or without parasitaemia). Significant positive association between age and reporting symptoms or displaying signs in the absence of parasitaemia. All p values <0.001.

The higher transmission amongst the S'tiếng might be expected to lead to less symptoms, particularly in older children and adults. Examining all age groups together, no difference between symptoms in smear positive individuals is evident between the ethnic groups (table 5.17). The higher proportion of smear negative S'tiếng reporting symptoms is noteworthy: whether this represents generally poorer health or intentional over-reporting of symptoms with a view to receiving more free medication is unclear, but either could introduce considerable noise into the relationship between smear positivity and symptomatology. None of the differences in documented fever are statistically significant.

Ethnic group	Any symptom		Any symptom other than headache alone		Temp>37.5 at time of survey		Total number	
	Smear neg	Smear pos	Smr neg	Smr pos	Smr neg	Smr pos	Neg	Pos
Kinh	26%	47%	16%	40%	3%	9%	6016	132
S'tiếng	**36%**	46%	**25%**	38%	4%	7%	7655	1285
Tày	31%	53%	19%	30%	1%	7%	375	30
Nùng	33%	49%	19%	35%	2%	5%	447	43
Total	31.8%	46.5%	21.1%	38.1%	3.6%	6.8%	14,493	1,490

Table 5.17. Symptomatology and ethnicity. Cell contents percentage of that ethnic group displaying specified symptom complex, by smear status. Bold figures indicate significant differences from other ethnic groups in the column (both $p<0.001$).

The relationships between symptoms and age amongst smear positive Kinh and S'tiếng subjects only are depicted in fig 5.17. Once again the decline of symptoms with age only becomes apparent once those reporting headache alone are excluded, and even then is only evident in the S'tiếng (mean age of symptomatic smear positive individuals 14.2yrs vs non-symptomatic smear positive individuals 18.8yrs, $p<0.001$), amongst whom a steeper decline with age might have been expected, and is not seen at all in the Kinh (24.1 vs 26.5yrs $p=0.34$). The decline of documented fever with age in the S'tiếng is more convincing (mean age pyrexial smear positive subjects 9.5yrs vs apyrexial 17.4yrs, $p<0.001$), although is again much less evident in the Kinh (24.2yrs vs 25.7yrs, $p=0.73$).

Significantly higher parasitaemias for *P. falciparum* were apparent in symptomatic individuals (table 5.18).

Symptom constellation	Geometric mean *Pf* parasitaemia		
	Asymptomatic	Symptomatic	p
Any symptom	1785	3080	0.003
Excluding headache only	1670	3895	<0.001
Pyrexia	2135	6470	0.001

Table 5.18. Parasitaemia and symptomatology

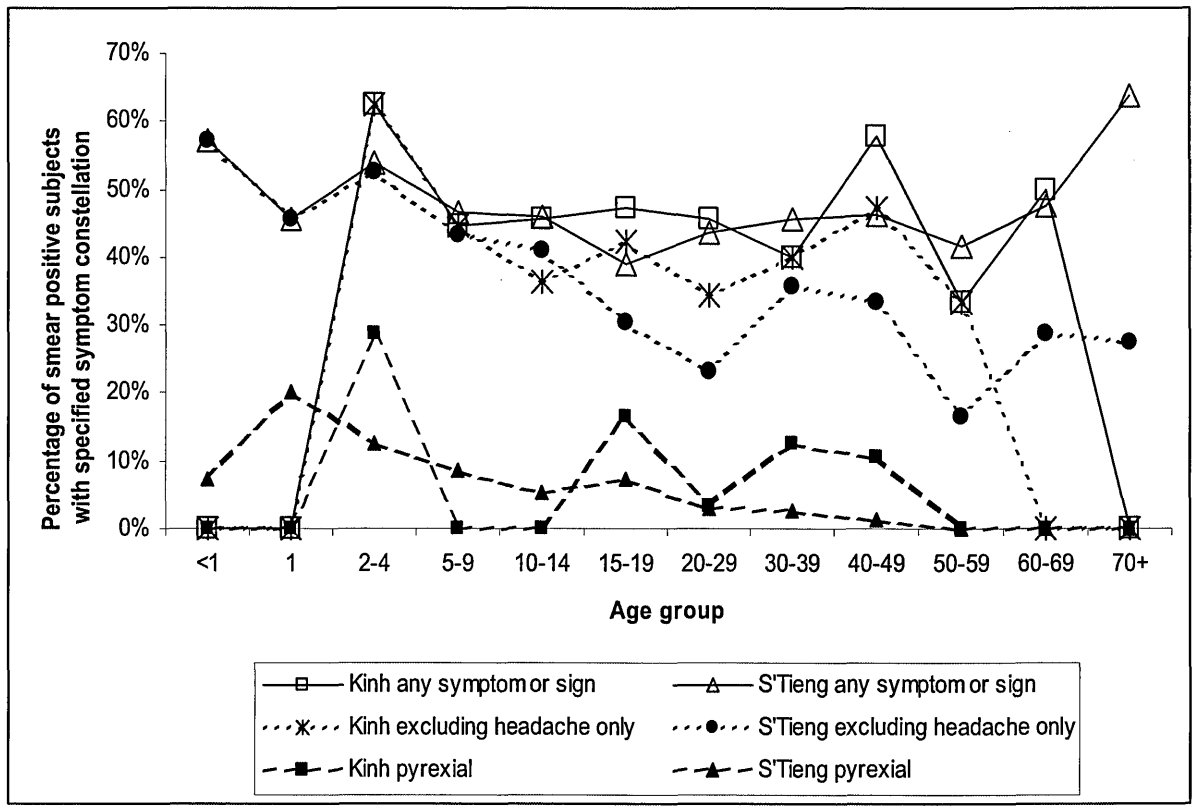


Fig 5.17. Symptomatology and age amongst smear positive Kinh and S'tiếng survey subjects.

The survey conducted in August 2000 included a follow up visit to all those who were smear positive at the time of the survey, in order to ascertain whether they had subsequently become unwell and were therefore presymptomatic rather than asymptomatic. Only one follow up visit was logistically feasible, and an interval of two weeks following the survey was felt to offer the best compromise between reasonable accuracy of recall and allowing sufficient time for symptoms to develop. 219 of 228 smear positive subjects were successfully followed up. The 9 individuals not followed up were not different in terms of age, sex or ethnic group from those in whom follow up succeeded. Only 102 individuals were followed up between 14 and 21 days of the survey, with 67 between 21 and 27 days, 39 between 28 and 34 days, and 11 between 35 and 41 days. The longer delays raise concerns over recall bias for the symptoms, and difficulty attributing measured splenomegaly or fever at follow up to the parasitaemia detected 5 or 6 weeks previously. Transmission is sufficiently low that the likelihood of acquiring a new, symptomatic infection in the interim is low, however. Rather than attempt to

generate a limit on the interval between the survey and follow up after which signs would not be attributed to the parasitaemia found during the survey, which would, inevitably, be somewhat arbitrary, all these individuals have been classed as symptomatic. Recall does seem to be a real issue, as the proportion of individuals reporting symptoms decreased with time since the survey (table 5.19). There were no significant differences in age or ethnic group between subjects followed up late and those followed at a reasonable interval.

Interval (days)	Fever	Rigors	Chills	Headache	Spleen	Pyrexia	Total number
14-20	49%	29%	20%	57%	38%	30%	102
21-27	28%	22%	19%	32%	11%	13%	67
28-34	27%	19%	14%	31%	20%	8%	39
35-42	0	0	0	0	0	18%	11
Total	37%	24%	18%	42%	24%	21%	219

Table 5.19. Interval between survey and follow up (in days) and symptomatology. Cell contents are percentage of individuals followed up in that time period with the specified symptom or sign.

Table 5.20 summarises the symptomatology of smear positive survey subjects. Headache alone does not seem to be a good indicator of symptomatic malaria in this population: whilst it remains statistically significant in univariate analysis, it quickly drops out in multivariate analyses, and, as demonstrated above, appears to have a different relationship with age than would be expected, and than other, better, clinical predictors of malaria. Therefore data classifying individuals with headache alone as asymptomatic at the time of survey or at follow up are also shown. The decline in the proportion of individuals reporting symptoms or displaying signs with increasing interval between survey and follow up raises concerns over either a recall bias, or incorrect administration of the questionnaire (addressing current or recent symptoms rather than those present at any time since the survey). Proportions of pre- and asymptomatic individuals extrapolated from the data on the basis that the pattern of symptoms seen in those followed up at 14-21 days from the survey is most likely to represent the true incidence of disease are also given. It is clear that at least half the individuals parasitaemic but asymptomatic at the time of the survey go on to develop symptoms over the ensuing weeks, although there remain a group of truly

asymptomatic individuals. Given the methodological shortfalls in the study, it is difficult to estimate the relative proportions of these groups.

Symptom complex / Symptom status	Any symptom	Symptomatic excluding headache alone	Extrapolated any symptom	Extrapolated excluding headache alone
Symptomatic	47%	40%	47%	40%
Presymptomatic	31%	28%	43%	38%
Asymptomatic	22%	32%	10%	22%

Table 5.20. Symptom status at survey and follow up according to specified symptom complex. Symptomatic indicates symptomatic at time of survey, presymptomatic indicates asymptomatic at time of survey, symptomatic at follow up. See text for other details. Extrapolated symptom complexes are based on extending the proportions of symptoms found at follow up in subjects visited 14-21 days post survey to those followed up later, in an attempt to correct for recall bias.

Ethnicity	Symptomatic	Presymptomatic	Asymptomatic	Total
Kinh	41%	21%	38%	29
S'tiêng	47%	**35%**[1]	**19%**[2]	174
Tày	40%	20%	40%	5
Nùng	62%	15%	23%	13
Other	50%	0	50%	2
Total	47%	31%	22%	223

Table 5.21. Symptom status by ethnic group (any symptom). Bold font indicates significant differences from other ethnic groups in that column. [1] p=0.036 [2] p=0.032.

Significantly fewer S'tiêng were asymptomatic than other ethnic groups, and significantly more were presymptomatic (table 5.21). These differences were much less pronounced when individuals with headache alone were classed as asymptomatic (table 11 appendix 2). There were no significant differences in age between the different symptom categories until those symptomatic with headache alone were excluded, by which definition the asymptomatic individuals are significantly older than the symptomatic subjects (p=0.002), which holds for the S'tiêng (p=0.004), but not the Kinh (p=0.75). There were no differences in symptom status between individuals harbouring *P. falciparum*, *P. vivax* or mixed infections at the time of the survey (table 12, appendix 2), and whilst there was a trend for decreasing initial parasitaemia with later and fewer symptoms, this was not significant (table 14, appendix 2). These result run counter to expectations, so the analyses

were repeated using hard evidence of disease in the form of documented fever or splenomegaly to classify individuals as symptomatic. The only differences to the pattern or significance of results was that individuals harbouring *P. falciparum* were more likely to be symptomatic at the time of survey than those harbouring *P. vivax*, but this difference disappeared when considering all symptomatic individuals (ie at time of survey or at time of follow up). The ethnic group relationships remained unchanged, and age relationships followed a similar pattern to those with symptomatology excluding headache. These analyses were complicated by incomplete spleen data at follow up for 24 individuals, but treating these as missing, or as no palpable spleen (as the field workers have insisted is the case) did not make a difference to any of the above associations.

Behaviour

The risk of malaria was increased by having received treatment for malaria or fever within the 2 weeks prior to the survey (OR 1.37 p=0.01). This effect was consistent whether all other subjects or only those who'd sought treatment were the comparator, and was not explained by any age or ethnic differences. Data on where treatment was sought was incomplete: 75% of treatment seekers had at least partial data collected, but in only half this number was the data complete (and a number of individuals did seek care from more than one outlet). Such data there are do not support any difference in risk of being parasitaemic at the survey between individuals who received treatment at a state health care outlet or a private health care outlet (whether medically staffed or not). Very few individuals used solely traditional remedies in the face of a fever. Data was not collected on the time elapsed between treatment and survey, thus it is not possible to estimate the proportion of those recently treated who might reasonably expected to be parasitaemic at the time of the survey even if they had received adequate treatment. The standard therapy for malaria in the study region is artemisinin derivatives, however, so parasite clearance would be expected to be rapid. Whether the association between recent treatment and

parasitaemia reflects inadequate treatment, poor compliance, or high risk behaviour and reinfection since treatment is impossible to say on the basis of this data, but the risk of reinfection within 2 weeks in the study area is very low. Recrudescence rates are high after short course artesunate monotherapy (Nguyen et al. 1993; Tran et al. 1994), the treatment most often administered, which may offer an explanation. The finding that smear positive individuals who'd received treatment for fever or malaria in the previous 2 weeks were more likely to be symptomatic than others (approx 85% vs 40%, $p<0.0001$) is more difficult to explain under this scenario, however, and might suggest inadequate treatment. Illness behaviour might also explain this phenomenon, and indeed when these subjects are compared with smear positive individuals who'd been recently treated for other reasons the difference in symptomaticity was less (approx 85% vs 65%, $p=0.012$), but still significant.

Bednet users were protected against malaria (OR 0.5 (0.42-0.60) $p<0.0001$). The S'tiếng were less regular users of bednets than other ethnic groups (90% vs 97%, $p<0.0001$). The odds ratio adjusted for ethnic group effects is 0.72 (0.60-0.86, $p=0.0004$).

A quarter of survey subjects were not born in the study area. Forty percent of these immigrants had been resident for over 10 years, however (table 5.22), and are likely to have similar pre-immunity as permanent residents. More recent immigrants have been classified by the malaria prevalence in their province of origin. Only crude slide positive rates were available from the National Institute of Malariology, Parasitology and Entomology, and even then the data are incomplete, but it was possible to assign an average of the slide positive rate over the 5 years prior to arrival in Phước Long to 1878 of the 2198 recent immigrants (table 5.23). The majority of immigrants are from low transmission regions, and would therefore be expected to have limited, if any, pre-immunity. It is thus a surprise to find a lower prevalence of parasitaemia amongst immigrants (4.2% vs 10.9%). There is a significant ethnic effect at work, however:

the S'tiêng are indigenous, whilst most of the Tày and Nùng and many Kinh are immigrants. Corrected for ethnic group, immigrants are more susceptible, an effect which is slightly more pronounced in those with a shorter duration of residence (table 5.24).

Years resident	Percentage (number)
<1	1.1% (40)
1	2.9% (108)
2	4.4% (166)
3	4.8% (180)
4	7.9% (298)
5	9.0% (337)
6	8.0% (300)
7	7.3% (276)
8	6.2% (232)
9	5.3% (200)
10	4.9% (185)
>10	38.4% (1445)

Table 5.22. Duration of residence of immigrants to Phước Long

SPR in province of origin	Proportion of recent immigrants
<2%	44.6% (869)
2-4.9%	25.2% (492)
5-9.9%	20.7% (403)
10%+	9.5% (185)

Table 5.23. Malaria prevalence in province of origin of immigrants resident in Phước Long for less than 10 years.

Ethnicity	Resident	Migrant	p	Resident >10yrs	Resident <10yrs	p
Kinh	1% (2877)	3% (3125)	<0.001	2% (4311)	4% (1630)	<0.001
S'tiêng	15% (8243)	7% (15)	0.384	15% (8247)	100% (1)	0.015
Tày	1% (84)	10% (276)	0.008	3% (150)	12% (205)	0.001
Nùng	3% (118)	10% (301)	0.021	3% (182)	12% (232)	0.019
Other	2% (65)	11% (56)	0.031	5% (20)	13% (15)	0.031

Table 5.24. Parasite prevalence by migrant status. Cell contents are percentage of ethnic migrant subpopulation smear positive (total number of subjects in ethnic-migrant category). Resident>10years includes permanent residents.

Haemoglobinopathies

The prevalence of haemoglobinopathies in the surveys has already been presented. Analysis of the relationship between HbE and parasitaemia in the surveys was not possible until individual haemoglobin types were made available to the investigators in late 2003, so the data presented here are from all surveys, including the February 2000 survey.

The malaria and haemoglobinopathy prevalence data indicate that ethnicity is likely to be a confounder of the relationship between the two: the S'tiêng and, to a lesser extent, the M'Nông, have the highest prevalences of both HbE and malaria. Table 5.25 displays the

variation of smear positivity with HbE genotype. HbE appears to be acting as a susceptibility trait in a number of ethnic groups, and the combined odds ratios, corrected for ethnic group, confirms this (table 5.26). Age does not seem to be an important confounder, as there were no significant differences in the prevalence of HbE at different ages, and correcting for age increases the effect slightly (table 5.27). α-thalassaemia increased the incidence of *P. vivax* infections in young children in Vanuatu, but there is no difference in species distribution between different genotypes (table 15, appendix 2), or in the prevalence of vivax or falciparum infections in individuals of different ages with different genotypes (table 16, appendix 2, data shown for S'tiếng only). It is possible that a number of individuals were bled in more than one survey, thus a handful of chronically infected HbE homozygotes sampled on multiple occasions might be biasing this result. Restricting the analysis to individuals sampled in Đak Nhau and Đồng Tâm (communes which were only visited in the first survey), and those positively identified as having attended only one survey, abolishes the significance of the effect, but leaves the magnitude and direction of the odds ratios unchanged (table 5.28).

HbE had no effect on parasitaemia at any age (fig 5.18a&b). Multiple linear regression of $\log_{10}$(*P. falciparum* parasitaemia) on age, HbE genotype (included either as an ordinal or two dummy variables) and ethnic group (as 5 dummy variables) revealed only S'tiếng and M'Nông ethnic groups and age as significant. Whilst HbE initially appeared to be associated with symptoms, this once again proved to be an ethnic artefact (table 5.29).

Ethnicity	AA	AE	EE	p
Kinh	5% (4961)	9% (184)	0 (1)	0.028
S'tiếng	21% (2040)	23% (1936)	24% (608)	0.156
Tày	5% (593)	0 (17)	0 (0)	0.325
Nùng	9% (522)	33% (9)	0 (0)	0.010
M'Nông	13% (346)	9% (202)	23% (31)	0.067
Other	6% (124)	7% (27)	27% (11)	0.032

Table 5.25. Percentage smear positive by ethnic group and HbE genotype (total subjects with genotype in ethnic subsample). p values from 3x2 χ^2 test for heterogeneity.

Genotype	OR (95% CI)	p
AE	1.12 (0.97-1.28)	0.118
EE	1.27 (1.03-1.56)	0.025
	Trend of odds	0.013

Table 5.26. HbE and smear positivity adjusted for ethnic group.

Genotype	OR (95% CI)	p
AE	1.17 (1.01-1.35)	0.034
EE	1.31 (1.05-1.63)	0.015
	Trend of odds	0.006

Table 5.27. HbE and smear positivity adjusted for ethnic group and age.

Genotype	OR (95% CI)	p
AE	1.12 (0.93-1.37)	0.234
EE	1.26 (0.93-1.70)	0.130
	Trend of odds	0.081

Table 5.28. HbE and smear positivity adjusted for ethnic group amongst individuals known to have been sampled once only.

Ethnicity	AA	AE	EE	p
Kinh	26% (4903)	27% (184)	0 (1)	0.758
S'tiêng	42% (2033)	41% (1932)	42% (608)	0.727
Tày	21% (588)	41% (17)	0 (0)	0.050
Nùng	21% (522)	56% (9)	0 (0)	0.014
M'Nông	27% (345)	33% (202)	45% (31)	0.061
Other	32% (123)	52% (27)	46% (11)	0.113
Total	29% (8551)	39% (2371)	42% (651)	<0.001

Table 5.29. Percentage of individuals reporting any symptoms (regardless of parasitaemia) by ethnic group and genotype. Total number in ethnic genotype subsample in parentheses.

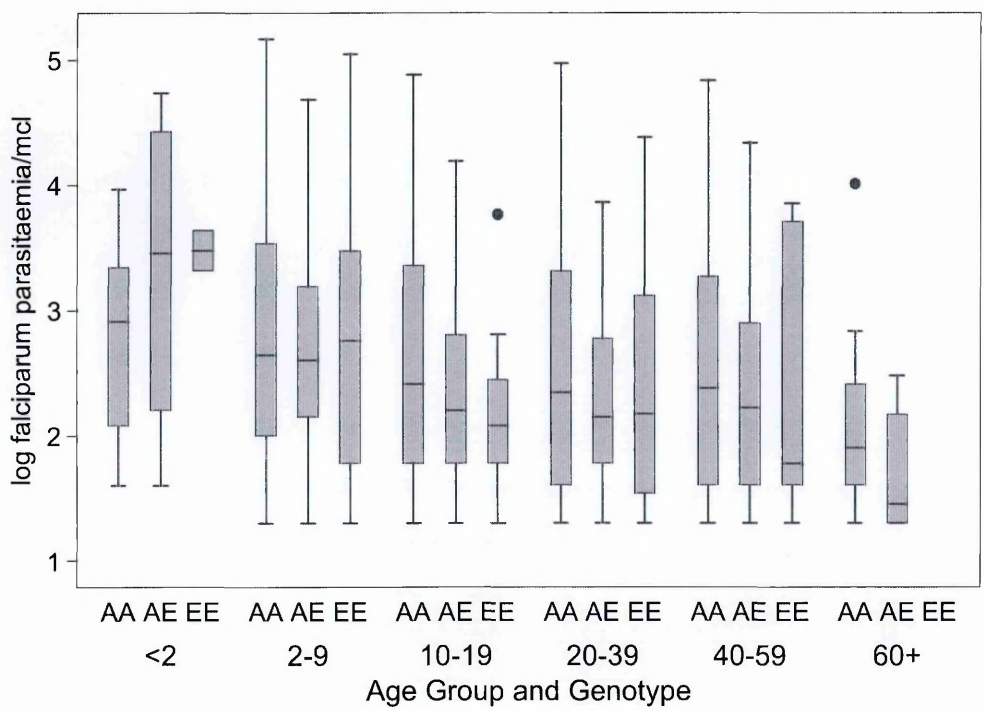


Fig 5.18a. Parasitaemia by age group and genotype

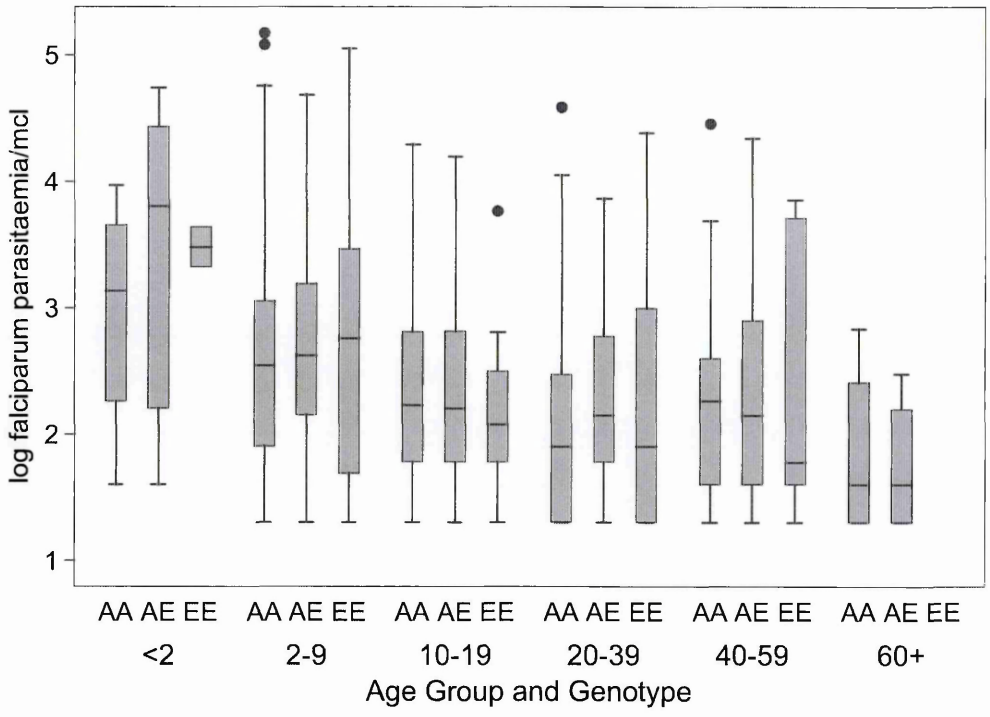


Fig 5.18b. Parasitaemia by age group and genotype in the S'tiếng

Discussion

Malaria transmission appears hypoendemic in the north of Phước Long district. There is little seasonal variation in prevalence, although the point prevalence estimates reported here were not sufficiently frequent to detect such fluctuations with any certainty, and reports from health care facilities would suggest seasonality in clinical disease incidence. The overall smear positive prevalence in these surveys was 9.3% (SE ±0.4%). These surveys probably overestimate malaria prevalence in the three communes as a whole, but constructing accurate sampling weights is impossible in the absence of good quality age and ethnic group breakdowns of hamlet populations. Constructing such corrections from the limited data available suggests the average prevalence over the duration of the study in the entire population to be 6.4%. There are fewer than expected well conducted, published prevalence surveys from the region with which to compare these data. A long running malaria investigation programme in Mae Sot on the Thai-Burmese border reported prevalences of between 1 and 6% in the mid 90's (Luxemburger et al. 1996), whilst a similar programme in Khánh Phú in the centre of Vietnam had seen prevalence fall to 15%

by 2000 (2001). Surveys in Khammouane province in southern Laos in 1995-6 revealed a prevalence of less than 1% in lowland villages but 5-10% in forest and foothill villages (Kobayashi et al. 1998), rising to 17% in 1997 (Toma et al. 2001), and anywhere between 5 and 35% in 1999 (Uza et al. 2002). There are even fewer data from Cambodia: workers crossing the border into Thailand had only 2.4% parasite prevalence, but a survey in a northeastern village revealed a prevalence of 45% (Trung et al. 2004). Reports from Burma are even sparser, but suggest transmission comparable to pre-control levels in other mainland Southeast Asian countries, with prevalences of 35-45% (Mya et al. 2002). Prevalence fell from 5% to 1.8% between 1999 and 2001 in Suối Kiet in Bình Thuận province in south central Vietnam (Erhart et al. 2004), whilst previously unpublished surveys in Vietnam conducted in 4 communes in the vicinity of Khánh Phú in 1998-9 demonstrated prevalences of 5, 8, 13 and 21%.

The relative proportions of *P. falciparum* and *P. vivax* are also in keeping with published reports, which usually describe *P. falciparum* as being responsible for 50-75% of parasitaemias, with the exception of the Thai-Burmese border, where the roles are reversed (Luxemburger et al. 1996; Panvisavas 2001). The relative excess of mixed infections contradicts those studies which have reported a relative dearth (Rosenberg et al. 1990a) and put this forward as evidence that *P. vivax* inhibited *P. falciparum*. Comparisons of speciation by light microscopy and PCR have usually revealed a significant number of missed mixed infections, and clinical evidence of cryptic dual infections abounds, suggesting that the apparent lack of *P. falciparum* and *P. vivax* coinfections may, in many cases, be artefactual. There are 3 possible causes for under detection of mixed infections: the parasitaemia of the missed species may be below the level of detection of light microscopy, the missed species is only present in ring forms, which are difficult or impossible to speciate, or identifiable forms of the missed parasite are present in such low levels that only exhaustive (and impractical) examination of the blood film would reveal their presence. *P. vivax* generally gives rise to lower parasitaemias than *P. falciparum*, and

on all three counts is therefore more likely to be missed. Population based PCR speciation studies have usually shown the expected, or greater than the expected, number of mixed infections (Mehlotra et al. 2000; Zhou et al. 1998). Recent surveys in Vietnam have tended towards the latter. Most speculation on *Pf*/*Pv* interactions has focused on the potential inhibition of one species by the other, rather than mutual potentiation, although at least one model predicts the possibility of such an effect (Mason et al. 1999). The excess of mixed infections is unlikely to be artefactual: over-detection of mixed infections is implausible (see above), and variation in the duration of parasitaemia between species would affect single species infections in the same way as the mixed, *unless* there are interactions between parasite species. Were only a subpopulation of the study subjects to be at risk from malaria, however, the contraction of the denominator would raise the probability of *Pf* or *Pv* infection in the population, increasing expected numbers of mixed infections. Assuming *Pf* and *Pv* to be transmitted in the same populations, and being otherwise independent, the calculated denominator from our data is 5155, suggesting that only 31% of observations were made in at risk individuals. If the number of mixed infections has been underestimated the population at risk would be even smaller. Repeating the exercise with the data for *P. falciparum* and *P. malariae*, and for *P. vivax* and *P. malariae*, yields estimates of the number of observations in at risk individuals of 2423 and 4788 respectively. These figures are likely to be less reliable than those for *Pf*/*Pv* due both to the smaller number of cases involved, and to the under recognition of isolated *Pm* infections as a result of their relative rarity and tendency of *P. malariae* to cause very low parasitaemias. All the factors affecting the detection of mixed infections mentioned above will also contribute to an underestimate of mixed infections involving *Pm*. A number of individuals were sampled in two or more surveys. It might be possible that a number of high risk, multiply infected individuals are artefactually raising the proportion with mixed infections. Re-estimating the population at risk using data from individuals positively identified as having only attended one survey reduced the projected

proportion at risk, however: 497 of the 8736 such subjects harboured *P. falciparum* and 331 *P. vivax*. The number of mixed *Pf/Pv* infections was 64, giving an estimated population at risk of 2570, or 29% of subjects. Extensive geographic and ethnic heterogeneity were apparent in the first survey, as was the expected effect of age. The association of these factors with parasitaemia might define a low or no risk population. The relative excess of mixed infections holds across most hamlets, ethic and age groups, however (tables 29 & 30).

Ethnic group	Age group												Total
	<1	1	2-4	5-9	10-14	15-19	20-29	30-39	40-49	50-59	60-69	70+	
Kinh	N/A	0	6.2	16.3	13.5	9.3	7.8	0	5.3	10.3	0	N/A	9.8
S'tiêng	4.8	1.8	2.3	1.6	1.6	1.3	1.9	2.9	3.3	3.1	7.3	0	2.3
Tày	N/A	N/A	N/A	0	10.1	17.5	0	4.9	N/A	N/A	N/A	21.0	6.9
Nùng	N/A	N/A	N/A	0	0	2.4	1.5	2.8	N/A	3.2	N/A	N/A	3.7
Total	7.4	3.1	3.7	2.8	3.0	2.4	2.6	2.7	4.1	4.1	9.8	5.7	3.4

Table 5.32. Ratios of observed:expected numbers of mixed *P. falciparum-P. vivax* infections by age and ethnic group

Hamlet		O:E ratio	Hamlet		O:E ratio
ĐẮC Ơ	Thôn 3	1.86	ĐỨC HẠNH	Ấp 3 Phú Văn	N/A
	Thôn 4	3.51		19 Tháng 5	N/A
	Thôn 6	2.40		Bình Đức 1	98.00
	Thôn 7	9.19		Bình Đức 2	N/A
	Thôn 9	6.81		Bù Ca Mau	6.34
	Bù Bưng	1.37		Bù Gia Phúc	1.22
	Bù Cà	1.28		Bù Gia Phúc 1	1.30
	Bù Khơn	1.84		Bù Gia Phúc 2	0
	Bù Xia	7.11		Bù Ca Roi	1.56
	Đak Lim	0		Đak Khâu	2.07
	Đak U	6.13		Đắc Sơn 1	3.40
ĐẮK IA	Thôn 2 ĐK	50.67		Đắc Sơn 2	3.81
	Thôn 3 ĐK	0		Đức Lập	6.94
	Thôn 4 ĐK	N/A		Khăc Khoan	N/A
	Thôn 5 ĐK	N/A		Thôn 4 Khăc Khoan	0
	Thôn 6 ĐK	N/A		Phước Sơn	N/A
	Bình Giải	2.17		Phú Nghĩa	0
	Bình Hà 1	2.71		Sóc 2 Căn	2.47
	Bình Hà 2	2.11		Sơn Trung	6.53
	Bình Tân	0		Thác Dài	N/A
	Bình Thủy	0		Tân Lập	N/A
	Bình Tiến	0			
	Bù Tam	2.75			

Table 5.33. Observed vs expected ratio of mixed *P. falciparum* and *P. vivax* infections by hamlet

Extensive variation in transmission between hamlets and ethnic group is a major feature of the study site. There appears to be a sampling effect in that the malaria ranking of different hamlets changes between surveys, but taking all surveys together it is possible to identify high and low risk hamlets. There is considerable confounding of this relationship by ethic group: each appears to have an independent affect, although formally testing this for individual hamlets proved impossible as all models containing dummy variables for ethnicity and hamlet failed to converge. Post hoc classification of hamlets into high, medium and low risk yielded a significant effect of both hamlet risk category and ethnic group in a logistic regression model of smear positivity on age, hamlet and ethnicity, but there was a significant interaction between being S'tiếng and living in a high risk hamlet to the extent that both became individually non-significant when the interaction term was added to the model. Geographic microheterogeneity in transmission is a well recognised phenomenon (see (Greenwood 1989) for a review) but ethnic variation of risk has been reported infrequently despite being an axiom of malariology in Southeast Asia. Many authors state that transmission is higher in the group under study, but give neither data nor reference (Lim 1992; Panvisavas 2001). The majority of published observations in the region have involved ethnic minority groups living in forested foothills (known areas of high entomological risk) separated from other ethnicities. The situation in Phước Long is different, in that the different ethnic groups live in close proximity to one another, and although the S'tiếng appear to live in "high risk" hamlets, there are no obvious ecological differences to explain the variation in transmission between these and "low risk" hamlets. The high transmission amongst the S'tiếng is apparent not only from the prevalence data, which might be biased by self selection, but by the more pronounced relationship between parasitaemia and age, with a younger peak than other ethnic groups, the younger age of the cases of severe malaria amongst the S'tiếng (see Chapter 7 – all S'tiếng cases were children, whereas most Kinh cases were adult), and the higher spleen rates in 2-9 year

old's (13.7% amongst the S'tiếng vs 2-3% among other ethnic groups except the M'Nông, with 10%). Little data exist on ethnic variation of malaria prevalence in Vietnam. A 1968 seroepidemiological survey in Bù Đốp (a commune adjacent to Đa Kia) revealed higher mean IFAT titres amongst the S'tiếng than the Kinh, although prevalence was similar (over 95%) (Colwell et al. 1971). The Khánh Phú group have consistently found higher prevalences in the Rac Lay than the Kinh (2001), and other reports of foci of very high transmission have come from ethnic minority villages, although without comparative data (Hung et al. 2005). Transmission appears to be higher amongst the S'tiếng than other ethnic minority groups in the area. Whilst insufficient data exist on relationship of parasitaemia with age and age range for severe disease amongst the Tày and the Nùng, not only is their prevalence lower than the S'tiếng, but the spleen rate in the 2-9 year old's is lower, and the gross discrepancy in prevalence between the sexes amongst adults of these ethnicities strongly suggests an occupational pattern of exposure, implying less transmission in and around the home. One obvious important factor is the larger proportion of S'tiếng not using bednets. Reported bednet use was generally very high, to the extent that there are only sufficient non-users to estimate their protective effect amongst S'tiếng subjects, where they are protective. Only 10% of S'tiếng reported not using a bednet, however, which is insufficient to account for the differences in prevalence.

Despite the apparently higher transmission amongst the S'tiếng, they remain as symptomatic as other groups. This is unexpected, as pre-immunity is expected to decrease the proportion of microscopically patent infections causing symptoms. Conversely there appear to be a significant proportion of parasitaemic Kinh subjects without symptoms, notwithstanding their low levels of prior exposure, which would be expected to result in every patent infection being symptomatic. The decline in symptoms with age is also less prominent than expected, and the difference in pattern between Kinh and S'tiếng less pronounced. Obvious possible confounders, such as age, have been explored and discounted above, and no clear explanations present themselves, although it remains

possible that deliberate over-reporting of symptoms in the expectation of receiving more medicine is commoner in the poverty stricken S'tiếng. Of note, significantly fewer parasitaemic S'tiếng were pyrexial than Kinh (7.7% vs 17.8%), and *P. falciparum* parasitaemia was slightly but significantly lower in the S'tiếng than the Kinh (log_{10} parasites/μl 3.40+/-0.03 vs 3.76+/-0.08, $p<0.0001$), and than other ethnic groups (log_{10} parasites/μl 3.40+/-0.03 vs 3.59+/-0.09, $p=0.0495$). The potential over-reporting of symptoms is clearly of concern in establishing the prevalence of asymptomatic infections in these populations: if all reported symptoms are included, as few as 10% of parasitaemic individuals are asymptomatic, compared to nearly a third if isolated headache is treated as being unrelated to the parasitaemia. Studies in Mae Sot found only 13% of episodes were asymptomatic, although precise definitions of symptoms were not given (Luxemburger et al. 1994; Luxemburger et al. 1997).

The prevalence of HbE is much higher in the S'tiếng than any other ethnic group other than the M'Nông (who only appeared in the first survey). It has been difficult to establish the effect of various red cell disorders on malaria by means of cross sectional surveys: no differences were found in such studies of α-thalassaemia, β-thalassaemia, G6PD deficiency or HbC. Only HbS has shown an effect on prevalence or parasitaemia in surveys. That HbE should appear to be a susceptibility factor for smear positivity was an unexpected finding. α^0 thalassaemia increased the chance of young children acquiring *P. vivax* malaria in Vanuatu (Williams et al. 1996), but no such species specificity is apparent here (table 5.32 – data from all surveys), neither does there appear to be an age specific effect (table 17, appendix 2). In addition to being a risk factor for harbouring parasites, HbE has no effect on the magnitude of parasitaemia, or on symptoms. It seems inconceivable that a genetic trait which has risen to such high frequencies in malaria endemic areas should act as a susceptibility trait for parasitaemia without ameliorating symptoms or parasite burden. The data have been exhaustively re-examined for possible errors, such as ethnic

misallocation, to no avail. The only potential confounder identified was a slight increase in the proportion of individuals with HbE living in higher transmission hamlets (table 5.33). Adjusting for level of transmission as well as ethnic group abolishes the significance of the effect of HbE and the trend across the genotypes, but has little effect on the magnitude of the odds ratios (and does not abolish significance if age is added as an adjusting factor). Even without these adjustments, the effect is not significant in the S'tiêng, who carry by far the greatest burden of HbE, though a trend to increasing prevalence with increasing copies of the β_E gene is still apparent. The only exception to this pattern is the M'Nông, in whom heterozygous HbE appears to protect (albeit not significantly), but even here, HbE homozygotes have an increased prevalence. On balance, these data clearly indicate that HbE does not protect against infection or clinical disease and does not appear to impair parasite multiplication *in vivo*, albeit within the limitations of a survey design to establish these effects. There is a suggestion that HbE may enhance susceptibility to malaria, but there are too many unresolvable issues with the data to assert this with confidence.

Ethnic variation in transmission in one African project has been attributed to putative genetic factors, having discounted entomological and behavioural differences, although these were not reported in detail (Modiano et al. 2001a). The remote possibility that an unknown susceptibility trait might explain the apparent effect of HbE is rendered even less likely by the absence of this effect in the S'tiêng. Detailed entomological and behavioural studies are required before postulating the existence of another, unknown genetic factor. The demonstration of negative epistasis between HbS and α thalassaemia (Williams et al. 2005) might provide a more plausible explanation, although the prevalence of α thalassaemia is low amongst the ethnic groups in which the effect is strongest. Hundreds, if not thousands, of survey samples would need to be genotyped for α thalassaemia to confirm or refute this hypothesis, however, and given the failure

to demonstrate any effect of haemoglobinopathies (other than HbS) on malaria indices in cross sectional surveys in the published literature, that effort does not seem justified.

Ethnicity	Species	Genotype		
		AA	AE	EE
Kinh	*P. falciparum*	2.4%	4.9%	0
	P. vivax	1.4%	3.3%	0
	Mixed *PfPv*	0.6%	0.5%	0
	Total number with genotype	4,959	184	1
S'tiếng	*P. falciparum*	11.4%	13.1%	14.0%
	P. vivax	6.7%	6.1%	6.4%
	Mixed *PfPv*	2.5%	2.9%	3.3%
	Total number with genotype	2,037	1,927	606
Tày	*P. falciparum*	2.7%	0	0
	P. vivax	2.0%	0	0
	Mixed *PfPv*	0.5%	0	0
	Total number with genotype	592	17	0
Nùng	*P. falciparum*	4.4%	0	0
	P. vivax	3.6%	33.3%	0
	Mixed *PfPv*	0.6%	0	0
	Total number with genotype	522	9	0
M'Nông	*P. falciparum*	8.7%	4.0%	9.7%
	P. vivax	3.8%	4.5%	12.9%
	Mixed *PfPv*	0.6%	0.5%	0
	Total number with genotype	346	202	31
Other	*P. falciparum*	2.5%	3.9%	27.3%
	P. vivax	1.6%	0	0
	Mixed *PfPv*	0	0	0
	Total number with genotype	122	26	11
Total	*P. falciparum*	5.0%	11.5%	14.0%
	P. vivax	3.0%	5.7%	6.6%
	Mixed *PfPv*	1.0%	2.4%	3.1%
	Total number with genotype	8,578	2,365	649

Table 5.32. Relationship between HbE genotype and plasmodium species, stratified by ethnic group. Cell contents are percentage of subjects from specified ethnic group with specified genotype who were found to be smear positive with the specified Plasmodium species or mixture of species.

Ethnic group	Genotype	Hamlet transmission level		
		Low	Medium	High
Kinh	AA	97%	96%	94%
	AE	3%	4%	6%
	EE	0	<1%	0
	Total number (%) in transmission tercile	2648 (52%)	1974 (38%)	517 (10%)
S'tiếng	AA	54%	48%	43%
	AE	39%	40%	43%
	EE	7%	12%	14%
	Total number (%) in transmission tercile	215 (5%)	1106 (24%)	3263 (71%)
Tày	AA	97%	95%	100%
	AE	3%	5%	0
	EE	0	0	0
	Total number (%) in transmission tercile	297 (49%)	168 (28%)	141 (23%)
Nùng	AA	97%	98%	99%
	AE	3%	2%	1%
	EE	0	0	0
	Total number (%) in transmission tercile	144 (27%)	200 (38%)	185 (35%)
M'Nông	AA	100%	60%	59%
	AE	0	36%	34%
	EE	0	4%	7%
	Total number (%) in transmission tercile	1 (<1%)	326 (57%)	243 (43%)
Other	AA	74%	88%	64%
	AE	26%	9%	22%
	EE	0	3%	14%
	Total number (%) in transmission tercile	23 (14%)	76 (47%)	63 (39%)

Table 5.33. Distribution of subjects bearing different HbE genotypes between hamlets of varying tercile of transmission, by ethnic group. Cell contents are percentage of subjects bearing specified genotype in specified transmission tercile within specified ethnic group, except Total rows, cell contents as described in table.

The decline of malaria over the period under study may have been due to one (or more) of five factors: the establishment of a high quality diagnosis and treatment centre in one of the communes (Đắc Ơ), the widespread use of artemisinin derivatives as first line antimalarials, the continuing distribution of bednets to vulnerable groups, ongoing deforestation reducing the habitat of Anopheles dirus, and the general improvement in the socioeconomic status of the country.

A malaria treatment centre was established in Đắc Ơ commune health station in 2000 as part of a series of treatment trials. Two local microscopists were trained, a new microscope provided and a supply of consumables ensured. A doctor from HTD was resident for most of the year. All individuals attending the health station with a febrile illness or other symptom constellation likely to be due to malaria were referred to the service, blood films were prepared and read, and treatment with artemisinin based therapy instigated. Individuals enrolled in one of the studies were asked to attend for follow up. Microscopic diagnosis had previously been available at the health station, and artemisinin based antimalarials provided by the national malaria control programme, but the presence of clinician, microscopist and drug was often sporadic. Although artemisinin followed by mefloquine is national policy, the latter remains expensive and is rarely available, so artemisinin alone is often used. The rapid fever clearance time will inevitably lead to patients curtailing the course, which, given that artemisinin needs to be given for 7 days to effect a cure, will inevitably lead to many recrudescences. Mefloquine or an equivalent was supplied to all patients enrolled in the treatment trials in Đắc Ơ. Some input was made to the health stations in Đa Kia and Đức Hạnh, but much less than Đắc Ơ: the resident microscopists were retrained at HTD in early 2001, and new microscopes were supplied towards the end of 2001. No antimalarials were supplied, however, nor were salaries met, and Đắc Ơ health station is too remote from the other two communes (a minimum of 20km on a very poor road) for an extended impact to be expected. Some additional funds were made available to the other health stations from early 2002 as part of their role in the cohort study, and this probably led to better malaria diagnosis and greater availability of the clinician thereafter. It is also possible that artemisinin derivatives have slowly replaced other antimalarials in informal outlets, as they have become more widely available and the price has dropped with their production in Vietnam. Given the remoteness of Đắc Ơ in particular, it may be that the widespread distribution of these drugs in the study sites only happened a short time before the surveys commenced, or even later. Artemisinin

derivatives are gametocidal (Kumar et al. 1990), and reduce the likelihood and/or duration of gametocytaemia (Chen et al. 1994; Targett et al. 2001; von Seidlein et al. 1998), thus reducing transmission to an even greater extent than a good diagnosis and treatment programme with other agents (Adjuik et al. 2004; Price et al. 1996), although there are very few such agents that could be considered adequate treatment in the study region.

Rolling out of bednets to the population in high transmission regions, and retreating nets, are ongoing activities of the National Malaria Control Programme. There was additional activity in Bình Phước province through the EC regional malaria control programme. It proved extremely difficult to establish details of bed net distribution during and immediately prior to the surveys, but it would appear that approximately 1000 bednets were distributed in Phước Long in 2000, and a further 500 in 2001. The bednets were apparently targeted at the poor and ethnic minorities. No data at commune or hamlet level was available. It is quite likely, however, that a number of the S'tiếng households involved in the surveys were the beneficiaries of this programme, and, as such, had only recently acquired their bednets. As the decline in prevalence was most marked amongst the S'tiếng, this may be very relevant. There was no trend to increased bed net use over the course of the surveys, however.

The border forests between Vietnam and Cambodia, particularly in the south, are under intense pressure from agricultural clearance (UNEP). The forest cover in Vietnam has halved since 1975, although specific data for the study zone was not available. Cleared forest may be planted with cashew trees, which may continue to provide a habitat for *An.dirus (Rosenberg et al. 1990b)*, or other crops such as cassava, coffee, pepper or maize, which do not. In both Đắc Ơ and Khánh Phú, sporozoite bearing *An.dirus* remain relatively plentiful in forest collections (M. Chambers– personal communication), presumably maintained by small groups of parasitaemic forest workers. Without quality

land cover data, not presently available, it is difficult to even speculate the magnitude of the impact of deforestation on malaria transmission in the study area.

Per capita GDP has risen steadily since the gradual relaxation of the Communist Party's hold on the economy which began in 1985. This process has been slow, inhibiting economic growth to a certain extent, but the lack of rapid expansion was probably a significant factor in the country being shielded from the impact of the Asian economic crisis. Most of the wealth has accumulated in urban areas, and most of the countryside remains poor, particularly more remote regions such as Phước Long. There have been improvements in income, nevertheless, although these have been at the mercy of fluctuations in international commodity markets: Vietnamese smallholders were hit hard by the slump in coffee prices between 1998 and 2001. The increased national wealth has also allowed for improved delivery of health care, although increased expectations have also led many health care workers to dedicate increasingly little time to their poorly paid health service responsibilities, and more to private work (often in the same facilities), so, with the exception of outreach programmes such as bednet treatment, this may have resulted in relatively little improvement in health care for the poorest members of society.

The slide positivity rate in Binh Phuoc province increased slightly from 4.9% to 6.3% between 1997 (the year the province came into existence) and 2001. Admissions for severe malaria to Phước Long hospital and HTD had been relatively stable in the late 90's. All the processes discussed above have been at work since at least 1995, apart from those activities related to the study. It seems likely, therefore, that the decline witnessed since 2000 has in part been due to these activities (in particular the treatment centre in Đắc Ơ, and the effect of the surveys as mass drug administration episodes), and the final rolling out of bednet provision and artemisinin therapies to the lowest echelons of society – the indigenous ethnic minority groups – in whom the decline has been greatest. The time course of the fall in prevalence follows that expected from some of the original models of

malaria eradication (Macdonald et al. 1964), and experiences with similar programmes in the region. Incidence of clinical and severe disease have fallen dramatically over the course of 2003 and 2004, but prevalence has now plateaued, suggesting the need for specific targeting of high risk areas and groups.

Conclusion

Malaria was hypoendemic in Phước Long at the time of the study. Transmission declined considerably over the four years under observation. There were no documented changes in bednet use, although there was a programme of bednet distribution coincident with the first two years of the study. The establishment of a treatment centre in Đắc Ơ would be expected to reduce transmission in the community served, but not have an effect in distant areas. A number of social and environmental factors, as well as the rolling out of artemisinin derivatives, would have contributed to a long term trend to declining transmission, but malaria morbidity had been static in the district during the late 1990's. The data gathered during this study unfortunately contributes nothing to establishing a cause for the fall in transmission.

P. falciparum was the dominant species, although a significant minority of parasitaemias were due to *P. vivax*. The excess of mixed infections is an interesting finding, which suggests either a hitherto unreported (albeit predicted in some models) positive interaction between the species, or that a large proportion of the study population was not at risk.

The surveys document considerable geographical microheterogeneity in transmission, consistent with data from other studies in the region. Mapping of households would have been an extremely useful adjunct to the surveys, but was both practically and politically impossible. Given the difficulties in identifying study subjects outlined in chapter 2, post hoc mapping of the hamlets is unlikely to be helpful.

The variation between surveys emphasises the difficulties in conducting malariometric surveys in low transmission regions. These surveys covered a wide geographic area and obtained a large sample size, but the resultant sampling strategy potentially lacked validity. A cluster survey would have had to used either a much smaller sample size or been restricted to a small geographic area. Not only would the former lack precision, but the geographic and ethnic variation documented in this survey would limit the validity of both designs: the latter study might give an unbiased estimate of the prevalence in one hamlet, but this would be meaningless just a few kilometres away.

The variation in malaria prevalence between different ethnic groups is the most striking finding of these surveys. The propensity of the minority groups to live separately results in considerable co-confounding of geography and ethnicity. Both ethnicity and geography appeared to exert independent effects. Detailed ecological and entomological studies would help explain these phenomena. The latter is ongoing in Đắc Ơ.

The ethnic groups with the highest prevalence of haemoglobin E were those with the highest prevalence of malaria. Adjusting for ethnic group there was no relationship between haemoglobin E and smear positivity, infecting species or parasitaemia. Similar findings have characterised cross sectional studies of malaria and most haemoglobinopathies other than HbS, even where case control or cohort studies have found quite a strong effect. It would appear that the degree of protection needs to be dramatic for prevalence to be affected. The high prevalence of α thalassaemia in the S'tiếng raises the possibility of an epistatic effect, but the enormous genotyping effort required to examine this possibility in these samples does not seem justified, given the difficulty in demonstrating any relationship between malaria and haemoglobinopathies in cross sectional surveys.

Chapter 6 – The KAP survey

Introduction

The gross disparity in malaria prevalence between different ethnic groups apparent in the cross sectional surveys requires explanation. Behavioural differences between Kinh and S'tiếng were cited as the most likely reason by the Vietnamese (Kinh) members of the project team. It was therefore essential to examine potential differences in malaria exposure prone behaviour. Such investigations are usually conducted by the administration of a knowledge, attitudes and practice (KAP) questionnaire.

The S'tiếng were said to make longer and more frequent trips to the forest than the Kinh, and be less likely to use bed nets or engage in economic activity other than farming. We set out to test the validity of these assertions and examine certain other risk factors which flowed from our knowledge of vector bionomics: the effect of house construction, the proximity of houses to forest or streams, and the sleeping habits of adults and children in the household. The KAP study was not carried out until 2003, by which time we'd been working in the region for 3 years. The striking difference between ethnic groups apparent to the project team was socioeconomic: the S'tiếng appeared much poorer, and much less likely to send their children to school. It had also been noted that although government programmes were in place to provide free treatment for certain conditions to ethnic minority individuals attending government health stations, these were often circumvented by local health care workers, and thus many S'tiếng preferred to attend private health care outlets where available. Cost of transport and the manner in which they were treated at the health station were cited as important factors in making this decision. As a result of these observations, the questionnaire was extended to examine economic status, educational achievement and health care seeking behaviour.

This survey was intended to examine differences in behaviour between individuals of different ethnic groups living in close proximity to one another. Whilst blood smears were taken from 2 members of each family, the survey was not powered to assess whether the behavioural traits measured were risk factors for malaria in the study population.

The methods have been covered in detail in Chapter 2, but in brief: hamlets with mixed ethnicity populations living in close proximity were selected. An ethnically representative number of families were chosen at random from the census list, and conveyed to local health workers to confirm that the selected families were still resident. The families were visited by teams from IMPE over a 2 week period, and the most senior member of the household sought. This individual was questioned about certain aspects of the house, and a table of all family members with a few behavioural details was completed. Two family members were selected to complete more detailed questionnaires about individual behaviour. Two individuals (not necessarily the same two) were bled for thick and thin blood smears. If families were not at home they could be replaced with families of the same ethnicity from a "reserve list" which had been randomly selected at the same time as the main list. The sampling method in Đắc Ơ was slightly different: three hamlets had already been sketch mapped as part of an ongoing longitudinal KAP. The hamlets had been selected to represent high, medium and low transmission microenvironments. An ethnically representative sample of households was selected at random using the same methods as the other hamlets, but there was no need for a confirmatory step. The questionnaires were administered over several weeks by the single individual responsible for the longitudinal KAP. Blood smears were not taken.

There was a considerable delay between the inception of the KAP survey and its implementation: it became clear during early 2001 that such a study would be highly desirable, an impression which gradually strengthened with the increasing conviction that the assumptions being made about the behaviour of different ethnic groups were not

necessarily valid. There was great reluctance to divert attention or resources away from the cohort study during 2001 and early 2002, however. Plans were revived in mid-2002, as the entomology study got underway. Preliminary meetings were held to discuss the KAP with IMPE-HCMC, but at this stage NIMPE expressed an intention to conduct a KAP study in parallel with the entomology study, so these plans were shelved. It became clear over the ensuing months that the NIMPE study was not going to meet all our requirements, so the plans were reactivated. The protracted sample confirmation process, the usual administrative machinations and the requirement for several trained teams to be available from IMPE-HCMC simultaneously then delayed the survey until late 2003.

This study was heavily dependent on the field workers, mostly drawn from IMPE-HCMC, guided by the local YTTB. My role was to initiate and design the study, design the study form in conjunction with Dr Mary Chambers and Dr Hung, plan and write the database with assistance from Hồ Vân Hiền, and attempt to supervise the conduct of the study, although this proved more difficult than previous surveys. Miss Tâm entered most of the data, and I analysed the results.

Results

Sampling success

Table 6.1 shows the percentages of visits completed in Đức Hạnh and Đa Kia. A smaller proportion of Kinh families were successfully visited than other ethnic groups. The disparity in expected and visited Tày-Nùng families in Bình Giải resulted from misattribution of ethnic group during sample selection rather than failure to visit selected families (many of the households thought, on the basis of name alone, to be Tày-Nùng turned out to be Hoa). Unfortunately S'tiếng families were substituted for non-S'tiếng families in a number of instances, and some families not on the reserve list were selected by the survey teams on the basis of unknown criteria to replace some absent households. This was inevitable in Thác Dài, where there were significantly fewer non-S'tiếng families

in our population list than in official contemporary data. We have no information other than assumed ethnic group on the families not visited, so cannot make any statements on their similarity to families successfully visited. The reason for visit failures are not clear for the majority of families, however the following general reasons were proffered as explanations of the ethnic group discrepancy in success rates: some Kinh families are included in a population list by dint of owning land in the hamlet, without being resident (although we had attempted to weed out these individuals through the pre-approval of sample lists by local health workers); a number of Kinh families have a temporary house they occupy in the hamlet during certain agriculturally determined seasons; many of the Kinh families, as well as most of the Tày and Nùng families, are recent immigrants to the region, and are more mobile than the indigenous S'tiếng.

No family in Đức Hạnh or Đa Kia refused to be interviewed for the survey, although some individuals did refuse capillary blood sampling for malaria diagnosis.

The census data was more accurate in Đắc Ơ, but only those families willing to participate in the longitudinal KAP, and likely to be resident throughout the planned one year duration of that study, were included in this KAP. Any household in the randomly selected sample not meeting these criteria was replaced by the nearest household of similar ethnicity not already in the sample.

Particular data disasters encountered were a significant number of households with no ethnic group information entered. These were not evenly distributed between the hamlets, unfortunately, and thus not evenly distributed between ethnic groups (based on names). Whilst it would have been possible to allocate a number of these families as S'tiếng, the remainder would not have been reliably apportioned to Kinh, Tày or Nùng, so this post hoc allocation was not performed.

Hamlet	Ethnic division	Sample size (% of planned hamlet sample)	Number (%) of sample successfully visited	Number substituted from reserve list	Number substituted not from reserve list	Post hoc ethnic allocation[1] (% of entire hamlet sample)	Actual reported ethnicity of households[2] (% of hamlet sample)
Bù Gia Phúc 1	Non-S'tiếng	19 (48)	15 (79)	4	0	19 (44)	Kinh 19 (44)
	S'tiếng	21 (52)	18 (86)	6	0	24 (56)	S'tiếng 24 (56)
Bù Gia Phúc 2	Non-S'tiếng	25 (60)	9 (36)	0	1	10 (27)	Kinh 10 (28)
	S'tiếng	17 (40)	20 (118)	7	0	27 (73)	S'tiếng 26 (72)
Đak Nhâu	Non-S'tiếng	32 (40)	25 (78)	0	0	25 (31)	Kinh 14 (30) Tày 1 (2)
	S'tiếng	49 (60)	49 (100)	7	0	56 (69)	S'tiếng 30 (65)
Thác Dài	Non-S'tiếng	32 (40)	16 (50)	0	0	16 (21)	Kinh 8 (14)
	S'tiếng	49 (60)	41 (84)	10	11	62 (79)	S'tiếng 46 (79)
Bình Giải	Kinh & other	19 (25)	15 (79)	0	0	15 (19)	Kinh 13 (18) Other 28 (38)
	Tày-Nùng	41 (54)	30 (73)	9	2	41 (51)	Tày 5 (7) Nùng 1 (1)
	S'tiếng	16 (21)	18 (113)	6	0	24 (30)	S'tiếng 19 (26)
Bù Tam	Kinh & other	22 (27)	17 (77)	0	0	17 (22)	Kinh 16 (21)
	Tày-Nùng	42 (51)	29 (69)	9	1	39 (51)	Tày 7 (9) Nùng 27 (36)
	S'tiếng	19 (22)	16 (84)	4	1	21 (27)	S'tiếng 20 (27)

Table 6.1. Visit success rate by household ethnicity. Note that with the exception of families in neither main or reserve list, the percentage of successful visits is based on originally attributed ethnic group, rather than ethnic group reported during the survey. Notes: 1) according to ethnic group putatively assigned during sample selection; 2) excluding mixed ethnicity households.

It became apparent that the question of whether individuals slept in the field or not was less straightforward than had been imagined. We know that individuals from many families sleep in plot huts near their fields if the fields are some distance from the house, or at certain critical points in the agricultural calendar, most notably harvest. It had been our intention to gather information about this sort of behaviour. A number of families, however, reported themselves as sleeping in the field, but added in the free text section that their main residence was "in the field". Whilst it might be argued that the location is more important than whether the house is the main residence (although nocturnal behaviour is likely to be different at the main residence), the classification of a house as being in the field may reflect a number of disparate realities. There are certainly a number of dwellings isolated from one another, surrounded by fields, and it does not seem unreasonable to classify these people with others who sleep in a field away from their main residence (if we have information on the type of sleeping quarters used by the latter). Other dwellings, however, clustered together in a population centre, might have small pepper or coffee fields adjacent to the house, and in this situation it would be inappropriate to class these individuals as sleeping in the field, when their next door neighbours without this adjacent cultivation are not. Although it was tempting to repeat all analyses reclassifying these families as not sleeping in the field, this information had been volunteered rather than specifically requested, so only those families for whom the interviewer chose to record that the house was in the field would have been excluded, thus introducing a potential systematic bias. The Hoa are a case in point: most households grow coffee, report their fields to be just a few minutes from the house, and report sleeping in a house under a bednet when they sleep at the field, yet none volunteered that their house is at the field.

Another issue was the application of a particular answer to all members of the family with a single pen stroke. Whilst it is entirely possible for this to be the case, it unfortunately suggests that information was not sought on each family member individually, as had been intended.

The most disappointing data collection failure was the poor labelling of the blood smears. Approximately 10% of the smears could not be associated with any individual in the list of family members (sadly containing a disproportionate number of smear positive individuals), further reducing the possibility of finding any association between behaviour and malaria in this survey.

Household demographics

Ethnic group

A total of 149 Kinh families, 251 S'tiếng, 29 Tày, 36 Nùng, 25 Hoa and 6 families of other ethnicity were interviewed. Many families thought to be Kinh or Tày-Nùng on the basis of name alone actually turned out to be Hoa, disrupting the expected numbers in Bình Giải hamlet. A further 37 families included individuals from 2 or more ethnic groups. The ethnic mix of these families is given in table 6.2. Ethnic group was not reported or attributable from the data gathered in 66 families.

	S'tiếng	Tày	Nùng	M'Nông	Khmer
Kinh	12	3	2	0	0
S'tiếng		1	1	2	1
Tày			7	0	0
Nùng				0	0

Table 6.2. Details of families of mixed ethnicity

Ethnic group data was not collected as part of the initial census, so we cannot determine how representative our sample was of the underlying population in this regard. It is possible to distinguish S'tiếng from non-S'tiếng on the basis of name. Although this distinction can fall down at the individual or household level, as Khmer and M'Nong ethnic groups sometimes use the same naming structure, very few members of these minority groups live in Phuoc Long, thus this technique is valid at the population level. The proportion of S'tiếng individuals in the KAP was the same as in the population (55% vs 55.4%), but the proportion of S'tiếng households was lower (41.6% vs 48.3%). This may reflect obsolete census data, as the number of S'tiếng households included in the

KAP was higher than anticipated due to the substitution of S'tiếng for non-S'tiếng households mentioned above, or may imply a disproportionate number of S'tiếng households amongst those with no ethnic group specified. In fact 62.5% of these families would appear to be S'tiếng, judged on surname alone, and if we assume this allocation to be correct, the percentages become 48.3% in the population and 48.7% in the KAP sample. Family size in the S'tiếng was slightly but statistically significantly larger than that in the census data (table 33, appendix 2), which may simply reflect unchecked population growth. Mean age and sex were the same in the KAP sample and the population (tables 34-35, appendix 2), though there were some statistically significant differences in age group structure of the population (table 36, appendix 2). Amongst the non-S'tiếng, these mostly balance when adjacent age groups are conflated, and are insufficient to suggest our sample is unrepresentative. Children are over-represented in the S'tiếng sample, which may reflect the birth rate, but this is unlikely to affect the results of the questionnaire. It is possible that it may reflect the smear results, and the analysis of the associations between malaria and behaviour, and we shall consider this below.

The household respondent was predominantly the head of house or his wife (table 37, appendix 2), and always (where recorded) a permanent member of the household. There were no inter-hamlet differences in respondent, although an adult child or other relative was more likely to respond in S'tiếng or Hoa households (table 38 appendix 2), possibly affecting the accuracy of certain data points, such as household annual income.

Primary outcomes

Bed net use

The overall percentage of households reporting using bednets regularly was 97%. The only individuals reporting never using bednets were S'tiếng, but even amongst this ethnic group, 90% used bednets all the time (table 6.3). The majority of households reported owning at least 2 bednets, although the state of repair varied widely (table 6.4). The larger

household size of the S'tiếng had a negative impact on the number of individuals sharing a bed net (table 6.5). On average 2 individuals would have to share a net in Kinh, Tày and Hoa houses, and 3 in S'tiếng and Nùng houses. Overall 10.5% of the bednets had been treated within the last 6 months, and 67% within the last year. The Hoa appeared worse than other ethnic groups at retreating their nets (table 6.6), but there was no significant difference between Kinh and S'tiếng in this regard.

Ethnicity	Never	Rarely	Sometimes	Always	Total
Kinh	0	0	0	682 (100%)	682
S'tiếng	**80 (5%)**	**28 (2%)**	**28 (2%)**	**1309 (91%)**	1445
Tày	0	0	1 (1%)	157 (99%)	158
Nùng	0	0	0	199 (100%)	199
Hoa	0	0	5 (4%)*	129 (96%)	134
Other	0	0	0	38 (1000%)	38
Total	80 (3.0%)	28 (1.1%)	34 (1.3%)	2514 (94.7%)	2656

Table 6.3. Reported frequency of bed net use in the home by ethnic group. Bold font indicates p<=0.001 vs all other ethnic groups and vs Kinh; *p=0.01 vs Kinh.

Number of nets in:-	total	good condition
0	4 (1%)	134 (23%)
1	74 (12%)	74 (13%)
2	232 (39%)	165 (23%)
3	184 (31%)	139 (23%)
4	70 (12%)	54 (9%)
5	22 (4%)	18 (3%)
6	10 (2%)	9 (2%)

Table 6.4. Total number of bednets per household and number of nets in good condition as assessed by interviewer (percentage of households).

Ethnic group	Mean good bednet index	Bednet per person (mean±SE)	Good bednet per person (mean±SE)	N
Kinh	0.89	0.63±0.02	0.57±0.03	149
S'tiếng	**0.65**	**0.45±0.01**	**0.31±0.02**	249
Tày	0.82	0.57±0.03	0.46±0.05	29
Nùng	**0.56**[1]	0.59±0.05	*0.35±0.07*[2]	36
Hoa	0.84	0.60±0.06	0.52±0.07	25
Mixed	0.68	0.56±0.04	0.40±0.05	37
Other	0.83	0.56±0.04	0.32±0.08	6
Total	0.74	0.53±0.01	0.41±0.01	531

Table 6.5. Indicators of condition and number of bednets per household by ethnic group (Good bednet index=Number of good condition bednets in house/Total number of bednets in house). Bold font indicates significantly different from all other ethnic groups, italic significantly different from Kinh. All p values<0.001 except: 1: p<0.001 vs Kinh, p=0.01 vs all other ethnic groups; 2: p=0.004.

Ethnicity	Never	Over 1 year ago	6-12 months ago	Within the last 6 months	Treated, but uncertain when	Total
Kinh	23 (16%)	22 (15%)	87 (60%)	12 (8%)	0 (0%)	144
S'tiếng	46 (20%)	21 (9%)	142 (61%)	21 (9%)	3 (1%)	233
Tày	3 (10%)	4 (14%)	22 (76%)	0 (0%)	0 (0%)	29
Nùng	8 (23%)	13 (37%)	14 (40%)	0 (0%)	0 (0%)	35
Hoa	**14 (67%)**[1]	5 (24%)	2 (10%)	0	0	21
Mixed	5 (12%)	4 (9%)	31 (72%)	3 (7%)	0 (0%)	43
Other	15 (60%)	6 (24%)	4 (16%)	0 (0%)	0 (0%)	25
Total	100 (20%)	70 (14%)	300 (59%)	36 (7%)	3 (1%)	509

Table 6.6. Bednet treatment by ethnic group. Cell contents are number (percentage) of households. 1: $p<0.001$ vs all ethnic groups & vs Kinh.

Bed net acquisition data was available for 498 families. Individuals from 15 families gave conflicting responses about the origins of their bednets in the household and individual questionnaires, so these households are not included in this analysis. Overall 39% of households were only using bought bednets, 24% using only freely distributed nets, and 37% had both distributed and purchased nets. There was significant ethnic variation in this distribution: S'tiếng households were much more likely to be relying on free nets, whilst Kinh families tended to use bought nets, and Hoa families supplemented their free nets with purchase ones (table 6.7).

Ethnic group	Mean retiring time	SE (mins)
Kinh	20:57	4.1
S'tiếng	20:24	3.3
Tày	20:22	6.9
Nùng	20:25	7.2
Hoa	20:18	9.2
Other	20:04	15.6
Total	20:33	2.3

Table 6.8. Mean retiring times by ethnic group. No significant differences.

Ethnic group	Only free	Bought and free	Only bought	Total
Kinh	23%	35%	**42%**	139
S'tiếng	**47%**	36%	17%	210
Tày	45%	38%	18%	40
Nùng	29%	49%	22%	45
Hoa	9%	**61%**[1]	30%	23
Other	33%	25%	42%	12
Total	36%	38%	26%	469

Table 6.7. Bed net acquisition by ethnic group. p values for percentage in bold vs percentage of that behaviour in other ethnic groups <0.001 except [1]$p=0.03$.

There were no significant differences in usual retiring time amongst the ethnic minority groups. The Kinh, however, went to bed on average 30 minutes later (table 6.8). Nightfall is typically around 6.30pm, and most individuals in rural areas will rise an hour and a half before dawn, so this represents approximately 15% more time exposed to anopheline bites.

Forest and field activity away from the home

Seventy five percent of households contained at least one individual who slept at the fields, and just over a third of all individuals were reported to sleep in the field at some point in time during the year (table 6.9). Although the Hoa appear to sleep out more often than other groups, the reliability of this data is uncertain (see note above). Those individuals interviewed in detail were only slightly less likely to be field sleepers (277/899 30%). Unsurprisingly 87% of the detailed interview group worked in the fields, thus it was interesting to note the difference in distance from house to field between the ethnic groups (table 6.10).

Ethnicity	Individuals sleeping in field % (n/N)
Kinh	31% (213/695)
S'tiếng	36% (556/1551)
Tày	41% (66/163)
Nùng	29% (57/199)
Hoa	67% (111/165)
Other	46% (22/48)
Total	36% (1025/2821)

Table 6.9 Percentage of individuals reported to ever sleep in the field (see text for caveats).

This difference is not only statistically significant, but could feasibly have an impact on malaria exposure: whilst most farmers in Vietnam rise very early, and set off for the fields just before dawn, the extra 30 minutes walk back from the fields in the evening could well take the S'tiếng deeper into *An.minimus* biting time. The difference in mode of transport may also have a bearing here: the S'tiếng are far more likely to have to make these journeys on foot (table 6.11).

Ethnic group	Foot	Bicycle	Motorbike	Total
Kinh	***71 (31%)***	17 (8%)	**138 (61%)**	226
S'tiếng	**241 (62%)**	16 (4%)	***135 (34%)***	392
Tày	***13 (31%)***	**9 (21%)**	20 (48%)	42
Nùng	22 (41%)	**12 (22%)**	*20 (37%)*	54
Hoa	*20 (50%)*[1]	2 (5%)	18 (45%)	40
Other	6 (55%)	0	5 (46%)	11
Total	374 (49%)	56 (7%)	336 (44%)	766

Table 6.11. Mode of transport to and from usual place of work by ethnic group. Bold font indicates significantly greater than, and bold italic font significantly less than, all other ethnic groups. Italic font significantly different from Kinh. All p values<0.005 except 1) p=0.03.

Ethnic group (N)	Distance to field (mean +/- S.D.)
Kinh (164)	14±18
S'tiếng (339)	**39±41**
Tày (40)	11±16
Nùng (48)	7±11
Hoa (40)	2±1
Other (11)	3±4
Total (643)	26±35

Table 6.10. Distance from respondents home to usual fields, measured in minutes taken to walk to field. Bold font indicates significantly greater than all and each other ethnic group, p<0.001.

Very few individuals were reported to spend any nights sleeping in the forest: a total of 18 individuals in 17 households. This represents 3% of Tày subjects, 1.2% Kinh subjects, 0.5% Nùng and 0.3% S'tiếng subjects, although the absolute numbers are far too small to make meaningful comparisons. Subsequent experience, gained primarily through the entomological surveys in Đắc Ơ, suggests that most forest workers are not local residents, and would thus lie outside the sampling frame for this survey.

Two thirds of those sleeping in the field reported using bednets whilst there. This proportion was similar between Kinh, S'tiếng and Tày, but significantly fewer Nùng and significantly more Hoa were bednet users (table 6.12).

Ethnic group	N	Number (%) of field sleepers using bednets
Kinh	220	131 (60%)
S'tiếng	533	343 (64%)
Tày	69	46 (67%)
Nùng	55	***13 (24%)***
Hoa	125	**124 (99%)**
Other	16	**15 (94%)**[1]
Total	1021	675 (66.1%)

Table 6.12. Bednet use amongst individuals reported to sleep in the field, by ethnicity. Bold font indicates significantly greater than, and bold italic font less than, all other ethnic groups. $p<0.001$ except 1) $p=0.006$.

House construction and situation

The majority of houses were constructed of bamboo or wood with corrugated iron or thatched roofs (table 6.13). A significant minority had walls made of brick, or tiled roofs. There was significant variation among ethnic groups (table 6.14). Kinh households were significantly more likely to be made of brick and have corrugated iron roofs, and significantly less likely to be made of bamboo and be thatched, whereas S'tiếng houses were significantly more likely to be made of bamboo and be thatched.

Material	Wall	Roof
Corrugated iron	4 (1%)	381 (64%)
Thatch	9 (2%)	133 (22%)
Bamboo	220 (37%)	0
Wood	301 (51%)	4 (1%)
Brick	50 (8%)	73 (12%)
Other	10 (2%)	4 (1%)

Table 6.13. House construction materials

Assuming brick to be a more complete barrier and offer fewer resting places than wood, which in turn is better in these regards than bamboo, and similarly corrugated iron roofs

being superior to tiles, which remain safer than thatch, the Tày have the most exposure prone house constructions, followed closely by the S'tiếng. Nùng and Hoa construction methods come next, although ranking these two groups with respect to one another would require quantitative assignment of risk to different materials. The Kinh have the safest houses.

Ethnicity	Total number	Wall construction material						Roof construction material				
		Metal sheet	Woven leaf	Bamboo	Wood	Brick	Other	Metal sheet	Thatch	Wood	Tile	Other
Kinh	145	1%	1%	***20%***	**62%**	**16%**	0	**83%**	***14%***	0	*3%*	1%
S'tiếng	246	0	2%	**45%**	***46%***[1]	***6%***[1]	1%	*54%*	**32%**	1%	13%	1%
Tày	29	0	0	52%	38%	10%	0	48%	31%	0	21%	0
Nùng	36	0	0	25%	67%	6%	3%	*42%*	17%	0	**42%**	0
Hoa	25	8%	0	44%	36%	0	12%	**100%**	***0***	0	0	0
Mixed	44	2%	0	36%	50%	9%	2%	66%	20%	0	14%	0
Other	5	0	0	40%	20%	20%	20%	80%	0	0	0	20%
Total	530	1%	1%	36%	51%	9%	2%	64%	23%	0%	12%	1%

Table 6.14. Roof and wall construction materials by ethnic group. Cell contents are percentage of houses in specified ethnic group. Bold font indicates a significantly higher, and bold italic significantly lower, percentage than all other ethnic groups. All p values <=0.005 except
1) p=0.03

The data on the distance from the house to the nearest field or forested area appears unreliable, in that, based on our experience, the answer will almost always be less than 100m, but in many instances the responses are in kilometres, suggesting that interviewers asked householders for this information, rather than estimating it themselves, and that householders gave the distance to their own fields rather than any adjacent field or plantation. Thus this data is not reported.

The information on the distance to the nearest stream may suffer from some of the same inaccuracies, but is still likely to represent the estimated distance to the nearest permanent stream, thus is reported. Overall 16% of households reported a stream within 50m, and a further 19% within 200m. The Nùng and the Hoa were significantly less likely to live near flowing water, assuming no data for distance to the nearest stream indicated no nearby stream (as the fieldworkers assured was the case). The other three ethnic groups were roughly as likely as one another to live within 200m of a stream (Kinh 36%, S'tiếng 41%,

Tày 38%), with the Tày non-significantly more likely to live within 50m (table 6.15). There was considerable variation between hamlets in the proportion of households situated close to a stream (appendix 2, table 39). The distribution of ethnic minorities by hamlet (table 40, appendix 2), suggests the hamlet variation does not fully explain the inter-ethnic variation, but this was impossible to test formally as a number of logistic regression models did not achieve convergence.

Ethnicity	Number of households	No nearby stream /no data	>500m	201-500m	51-200m	<=50m
Kinh	146	56%	7%	2%	17%	18%
S'tiếng	249	***44%***	4%	11%	26%	14%
Tày	29	55%	3%	3%	10%	28%
Nùng	36	**83%**	0	0	11%	6%
Hoa	25	**96%**	0	0	4%	0
Mixed	44	55%	7%	5%	7%	27%
Other	5	40%	0	0	40%	20%
Total	534	54%	5%	6%	19%	16%

Table 6.15. Distance from house to nearest stream by ethnic group. Bold font indicates significantly greater, and bold italic font significantly smaller, proportion than all other ethnic groups (but assumes no data=no stream). All p values <0.001 except S'tiếng vs Kinh for no stream p=0.02.

Ethnicity	Any animal	Cattle or pigs	Pigs	Cattle	Chicken
Kinh	50 (34%)	36 (24%)	20 (14%)	16 (11%)	20 (14%)
S'tiếng	105 (42%)	**92 (37%)**	**65 (26%)**	37 (15%)	15 (6%)
Tày	8 (28%)	6 (21%)	4 (14%)	2 (7%)	3 (10%)
Nùng	19 (53%)	**18 (50%)**	6 (17%)	**15 (42%)**	0
Hoa	**15 (60%)**[1]	***1 (4%)***[1]	***1 (4%)***[1]	***0***	15 (60%)
Mixed	19 (43%)	8 (22%)	7 (16%)	4 (9%)	7 (16%)
Other	3 (60%)	0	0	0	3 (60%)
Total	219 (41%)	161 (30%)	103 (19%)	74 (14%)	63 (12%)

Table 6.16. Livestock ownership by ethnic group. Cell contents are number of households reporting owning animal (% of households of that ethnicity). Cattle or pigs shown as separate column as denominator unknown for chicken, and hence any animal, ownership (see text). Bold font indicates significantly greater, and italic significantly smaller, proportion than other ethnic groups. All p values <0.01 except 1) p=0.05. Chicken ownership not formally analysed as denominator unknown.

Only 198 households reported owning animals, which seems to underestimate our experiences. The vast majority of the animals were kept in or near the house, with only 24 households reporting pens greater than 20m away from the house, only 3 of which were

greater than 50m from the house. Details of animal ownership are presented in table 6.16. Ownership of pigs or cattle was the focus of specific questions, whereas the ownership of chickens was volunteered in a free text field, thus the denominator is uncertain. Thus although more Hoa and fewer S'tiếng owned chicken than other ethnic groups, the validity of this observation is uncertain. More S'tiếng households owned pigs than others, and more Nùng owned cattle, contributing to both of these groups having a higher proportion of livestock owners overall for the types of animals specifically enquired about.

Health care seeking behaviour

Information on health care seeking behaviour was available from both the household and individual questionnaires. The overall proportions of households using different facilities are shown in table 6.17. The "depends on illness" category was attached to a free text field intended to capture real health care seeking behaviour, which will usually depend on perceived severity of symptoms. This field was invariably blank, limiting the value of the data collected through this set of questions. There were ethnic differences in behaviour, but the hamlet effect seems more important (table 45, appendix 2 and table 6.18). As the hamlets with high hospital usage are all in the commune closest to the hospital, and those with highest health station use in the most remote commune, distance is clearly one factor. The relatively high utilisation of private doctors in the hamlets in Đa Kia is not readily explained by distance, however, as the health station is close to at least one of these hamlets. The Hoa, Tày and Nùng populations are all relatively geographically circumscribed, thus the only inter-ethnic difference amenable to examination across all three communes is that between Kinh and S'tiếng (table 6.19). None of the differences are significant, although there would appear to be a trend towards the S'tiếng utilising more public health services and less private services. We also questioned householders about health care seeking behaviour if a child

Health station	149 (25%)
Hospital	93 (16%)
Private doctor	143 (24%)
Depends on illness	184 (31%)
Other	22 (4%)

Table 6.17. Overall household health service utilisation

was ill rather than an adult. There was a small and non-significant trend towards attending the health station regardless of illness (appendix 2, table 41).

Village	Number of households	Health station	Hospital	Private doctor	Depends on illness	Other
Đắc Ơ	198	39%	1%	3%	56%	1%
Đức Hạnh	236	20%	37%	19%	23%	1%
Đa Kia	157	15%	3%	59%	12%	11%
Total	591	25%	16%	24%	31%	4%

Table 6.18. Inter-commune variation in health service utilisation

Village	Number of households	Health station	Hospital	Private doctor	Other
Đắc Ơ	87	**90%**	***1%***	***7%***	2%
Đức Hạnh	182	***26%***	**48%**	***25%***	***2%***[1]
Đa Kia	138	***17%***	***4%***	**67%**	**12%**
Total	407	37%	23%	35%	5%

Table 6.18a. Inter-commune variation in health service utilisation for families giving definitive answer. Bold font indicates significantly greater, and bold italic font significantly fewer households utilising that health care provider than in other villages. All p values <0.001 except 1) p=0.003.

The data from the individual questionnaire obviously relates only to adults, and is rather more restrictive in scope, addressing the question of where health care was sought if an individual fell ill whilst "working in the field". The combination of most households' fields being near the house and the lack of clarity in establishing that the focus should be on episodes of illness occurring away from the home has, in most cases, resulted in an assessment of adult health care seeking behaviour. Tables 6.20 & 6.21 detail the breakdown of these behaviours by village and ethnic group. They confirm the dominance of a geographical effect over ethnic variation, the central role of the health station in Đắc Ơ, and the private sector in Đa Kia. The hospital appears less dominant in Đức Hạnh than indicated by the household data. Although the S'tiếng appear to be more inclined to self treat than other ethnic groups, this apparent effect is skewed by a large sample in Thác Dài, and may not be representative (table 40, appendix 2). Attempts to compare Kinh and S'tiếng were also hampered by the concentration of observations in just a few hamlets, but no obvious differences were discernible except in Thác Dài.

		Number of households	Health station	Hospital	Private doctor	Depends on illness	Other
Thôn 4	Kinh	17	53%	0	6%	41%	0
	S'tiếng	31	32%	0	0	68%	0
Thôn 7	Kinh	30	37%	3%	3%	57%	0
	S'tiếng	6	67%	0	0	33%	0
Bù Bưng	Kinh	19	37%	0	5%	58%	0
	S'tiếng	44	34%	0	2%	61%	2%
Đak Khâu	Kinh	14	0	57%	36%	7%	0
	S'tiếng	30	17%	53%	27%	3%	0
Bình Giải	Kinh	13	15%	0	62%	23%	0
	S'tiếng	20	15%	0	65%	20%	0
Bù Tam	Kinh	16	0	0	**75%**	13%	13%
	S'tiếng	21	19%	0	14%	5%	**62%**
Bù Gia Phúc 1	Kinh	19	0	11%	21%	68%	0
	S'tiếng	24	4%	21%	4%	71%	0
Bù Gia Phúc 2	Kinh	9	33%	11%	11%	33%	11%
	S'tiếng	26	58%	31%	8%	4%	0
Thác Dài	Kinh	7	14%	43%	14%	29%	0
	S'tiếng	45	16%	40%	27%	16%	2%
Total	Kinh	144	23%	10%	24%	41%	2%
	S'tiếng	247	26%	19%	16%	33%	6%

Table 6.19. Differences in health service utilisation between Kinh and S'tiếng households in different hamlets. Significant differences are shown in bold.

Village	Self treat at home	Self treat in field	Health station	Private Doctor	Hospital
Đắc Ơ	*7 (7%)*[1]	0	**86 (91%)**	*1 (1%)*	0
Đức Hạnh	**34 (24%)**	1 (1%)	*38 (27%)*	44 (31%)	**23 (16%)**
Đa Kia	*1 (1%)*	1 (1%)	*20 (29%)*	**37 (54%)**	10 (14%)
Total	42 (14%)	2 (1%)	144 (48%)	82 (27%)	33 (11%)

Table 6.20. Individual healthcare seeking behaviour on falling ill at work in the field, by village. Cell contents are number choosing that health care provider (% of valid responses). Bold font indicates significantly greater, and bold italic significantly smaller, proportion than other villages. All p values <=0.005 except 1) 0.03.

Ethnic group	Self treat at home	Self treat in field	Health station	Private Doctor	Hospital
Kinh	*2 (3%)*	0	37 (60%)	16 (26%)	7 (11%)
S'tiếng	**31 (23%)**	0	71 (52%)	*28 (21%)*[3]	*6 (4%)*
Tày	2 (14%)	0	7 (50%)	3 (21%)	2 (14%)
Nùng	0	0	4 (100%)	0	0
Hoa	*0*	1 (3%)	**12 (32%)**[2]	14 (38%)	**10 (27%)**
Other	0	0	**1 (11%)**[1]	**8 (89%)**	0
Total	36 (14%)	1 (0%)	132 (50%)	69 (26%)	25 (10%)

Table 6.21. Individual healthcare seeking behaviour on falling ill at work in the field, by ethnic group. Cell contents and indicators of significance as table 6.20. All p values<=0.005 except 1) 0.01 2) 0.02 3) 0.04.

Economic status

Annual income data was reported from 491 families (72%). Families lacking this information were more likely to be S'tiếng (25% of S'tiếng households) or Kinh (20%) than other ethnic groups. Income data was missing from 11% of houses of unknown ethnic group. Reported household annual income ranged from 100,000 Vietnamese Đồng (VNĐ) to 150,000,000VNĐ (figs 1 and 2 and table 6.22). The distribution is, as expected, markedly skewed to the right, and a "rounded estimate" effect is evident with peaks at 10, 15, 20, 25, 30 and 40 million, with a noticeable paucity of households reporting 9 or 11 million, and a near absence of families reporting other values over 10 million except at 12 million – which presumably represents a calculated value from a response of "about a million a month". Values at both ends of the distribution are difficult to believe. The financial context is an average monthly *wage* in Vietnam of 650,000VNĐ (approximately US$43 – dollar value quoted as VNĐ-USD rate reasonably constant, whilst VNĐ-GBP exchange rate depends on GBP-USD rate). The government definition of extreme poverty sufficient to qualify for additional state benefits is total household earnings of less than 50,000VNĐ per month. In addition to the various and diverse incentives to misreport household income, the large powers of ten involved in dealing with the currency are prone to transcription errors. The majority of households reporting incomes of less than a million Đồng per year probably fall into this category: five of the nine families report owning a motorbike, and 7 report farming cashews, likely to bring in more than a million Đồng per year by itself. The other obvious transcription error candidates are those earning over 10 or 100 million Đồng, but we have less corroborative data here. Nineteen of the 186 families earning over 10 million đồng/year described themselves as having insufficient money to meet their needs, and specified a number of months during which they did not have enough rice for the family. Whilst this may hint at transcription error, these measures are much more subjective than the ownership of a motorbike.

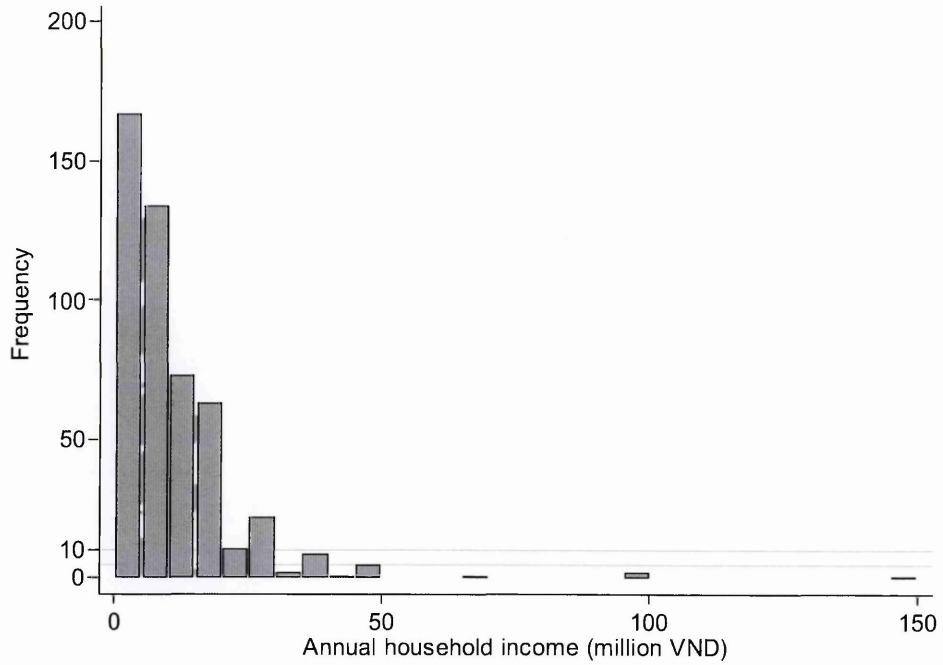


Fig 6.1. Overall household income distribution

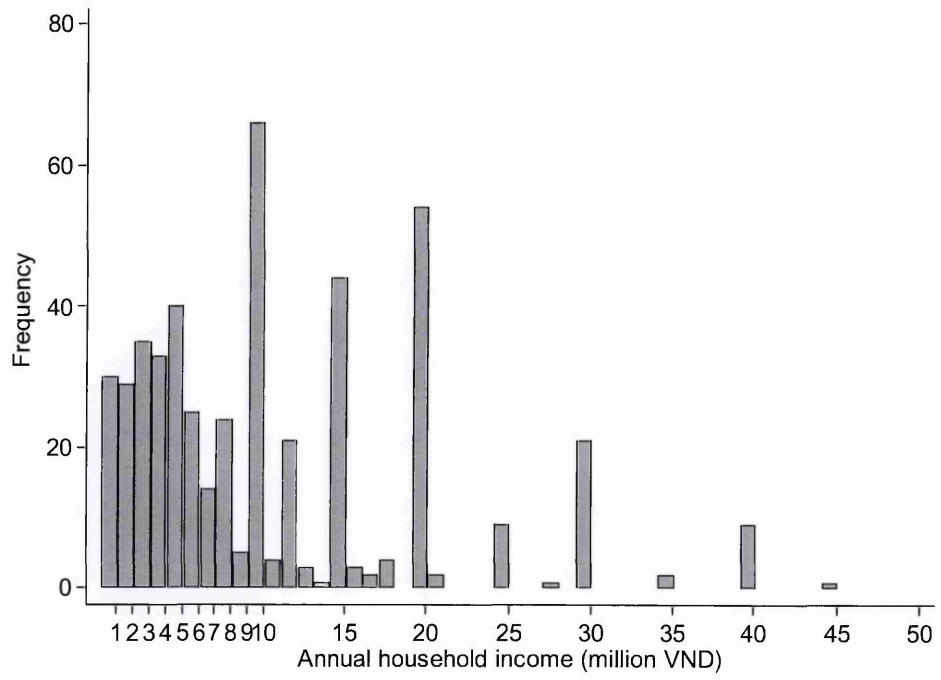


Fig 6.2. Income distribution for households earning less than 50 million Đồng per year

Mean +/- SE	12.16+/-0.587
Median (interquartile range)	10 (4-15)
Range	0.1-150
Mode	10

Table 6.22. Summary of annual household income data: all numbers are in million VNĐ

Table 6.23 summarises a number of the indicators chosen to characterise a household's wealth, and their relationship to recorded annual income. The majority of households described owning land. Whilst land ownership can be a somewhat tenuous concept in these highland regions, the implication is that these households are working their own land, rather than labouring for others. Cultivation of rice and cassava are associated with lower annual income, whilst pepper and coffee are associated with greater income. This is not surprising as rice is very much a subsistence crop in Phước Long, with very few areas of productive paddy. The fact that cassava cultivation is not significantly associated with greater poverty is slightly more surprising, as it is traditionally the lowest rung on the food ladder in Vietnam. It is easy to grow, however, and has become a major raw material in monosodium glutamate production, which may account for its popularity across the income spectrum. Pepper remains a valuable cash crop, whilst the volatility of coffee prices means that only wealthier households can afford to keep coffee on their lands. Cashew production has become a major source of income for many households in diverse ethnic groups in the district, and is no longer discriminatory. That cattle or pig ownership is not predictive of higher household income is surprising. The significant ethnic differences in livestock keeping preferences noted above, which may be culturally determined, are likely to explain this observation. Ownership of a motorbike or television remains the privilege of the slightly better off. The subjective opinion of whether a household is earning enough money is unsurprisingly linked to income, as is the semi-objective indicator of the number of months a household doesn't have enough rice to feed all it's members. The questions surrounding rice buying habits of the household sprung from a successful project run in the Mekong Delta, which found these patterns to give a useful indicator of a family's purchasing power. Poorer families could only afford to buy small quantities of rice at one time, and thus had to buy them often, whereas wealthier families bought large quantities infrequently. There was no association between annual

income and frequency of buying rice, quantity purchase each time, or estimated quantity of rice purchased each year in our sample.

	No	Yes	p
School educated	5 (3-10)	10 (6-20)	0.0001
	7.7+/-0.6	14.3+/-0.8	
Enough money	4 (2-7)	15 (9-20)	0.0001
	5.5+/-0.3	16.7+/-0.9	
Own bicycle	10 (4-16)	8 (4-15)	0.3844
	12.0+/-0.6	12.6+/-1.6	
Own motorbike	5 (2-10)	10 (5-20)	0.0001
	5.9+/-0.5	14.1+/-0.7	
Own TV	5 (3-10)	11 (8-20)	0.0001
	8.1+/-0.6	15.3+/-0.9	
Own radio	10 (4-15)	12 (5-20)	0.0341
	11.8+/-0.6	14.1+/-1.6	
Farm cashew	7 (3.5-15)	10 (4-15)	0.1439
	10.7+/-1.2	12.4+/-0.7	
Farm pepper	7 (3.5-12)	15 (8-20)	0.0001
	9.6+/-0.5	17.7+/-1.5	
Farm rice	10 (5-20)	7.5 (4-15)	0.0193
	13.6+/-0.9	10.3+/-0.6	
Farm cassava	10 (4-18)	8 (5-15)	0.2745
	12.9+/-0.8	10.3+/-0.7	
Farm coffee	8 (4-15)	15 (10-30)	0.0001
	10.3+/-0.5	21.3+/-2.4	
Own cattle	9 (4-15)	10 (5-20)	0.0691
	12.1+/-0.7	12.6+/-1.0	
Own pigs	10 (4-15)	11 (5-20)	0.0939
	12.0+/-0.7	12.7+/-1.0	
Any month without rice	15 (9-20)	4 (2-7)	0.0001
	16.6+/-0.9	5.4+/-0.3	

Table 6.23. Annual income (million VNĐ) and other household wealth indicators. Top row for each criterion is median (interquartile range) and bottom row is mean+/-SE. Given highly skewed distribution, comparisons have been made by non-parametric means.

Ethnic group	Annual income		Number
	Mean +/- SE	Median (25%-75%)	
Kinh	17.0+/-1.5	**12 (8-20)**	116
S'tiêng	8.5+/-0.6	***6 (3-11)***	187
Tày	11.7+/-1.9	10 (5-20)	27
Nùng	10.3+/-1.3	8 (5-15)	35
Hoa	21.8+/-5.7	**15 (10-20)**	25
Mixed	12.4+/-1.7	10 (5-20)	38
Other	26.2+/-5.5	25 (16-30)	5
Total	12.2+/-0.6	10 (4-15)	491

Table 6.24. Household annual income (million VNĐ) by ethnic group. Bold font indicates significantly higher, and bold italic significantly lower, income than all other ethnic groups. All p values <0.005.

Interethnic variation in annual income is displayed in table 6.24, and table 6.25 shows the distribution of the secondary economic indicators in different ethnic groups. The small group of Hoa families reported the highest average annual income, followed by the majority Kinh, both groups earning significantly more than other ethnic groups. Tày and Nùng households reported approximately average incomes (although the latter earned significantly less than the Kinh), with the S'tiếng the poorest, reporting significantly lower annual earnings than any other group. The secondary economic indicators broadly followed the patterns discerned above, with a few notable anomalies. The most striking of these is the Nùng: second from bottom in terms of reported annual earnings, but second only to the Kinh in the proportion of households reporting sufficient money for household needs and sufficient rice for the whole year. Two possible explanations present themselves: the Nùng are the most likely to grow pepper (which remains a highly lucrative cash crop) and own cattle, thus they must be earning more than they report. Alternatively, the high bicycle and radio ownership (as opposed to motorbike and TV), and greater tendency toward cassava and rice cultivation might point to a more traditional way of life, self sufficient in most aspects, with pepper as the cash crop and cattle conferring status. The S'tiếng would appear to have relatively high motorbike and pig ownership for their lowly income status. The latter may relate to the important role of pigs in S'tiếng culture, but the former does run contrary to expectations, and defies easy explanation. The high reported earnings of the Hoa sit at odds with their relatively low level of perceived financial sufficiency. One possible explanation is their universal adoption of coffee as a cash crop: the recent drastic fall in coffee price inflicting considerable hardship on those who'd based their farming strategy on the bean. Concerned that the Hoa's behaviour might have confounded the relationship between income and coffee growing, we repeated the analysis excluding the Hoa. This made no difference to the existence or statistical significance of this relationship. Whilst the Tày household's income, household construction (greater use of cheaper materials) and secondary income indicators (low

motorbike ownership, relative financial insecurity and staple food insufficiency) would appear to be consistent, the a priori expectation was that the Tày would stand nearest the Kinh in terms of wealth and behaviour. That this is not the case may reflect a small, unrepresentative sample, or an incorrect assumption.

Ethnicity	Number	Enough money	Bicycle	Motorbike	TV	Radio	Any month without rice	Mean months without rice
Kinh	146	**82%**	***18%***[1]	**81%**[1]	**66%**	19%	*17%*	*0.7+/-0.2*
S'tiếng	247	***33%***	23%	73%	*46%*	***12%***[1]	**66%**	**2.9+/-0.2**
Tày	29	52%[+]	31%	***59%***[1]	59%	14%	48%*	1.7+/-0.5*[2]
Nùng	36	**81%**	**67%**	***61%***[1]	56%	**42%**	*19%*	*1.0+/-0.4*[2]
Hoa	24	67%	12%	84%	**80%**[2]	4%	28%	1.4+/-0.5
Mixed	44	**80%**	14%	84%	64%	14%	21%	1.1+/-0.4
Other	5	**100%**[1]	0	80%	40%	0	***0***[1]	0
Total	531	57%	23%	75%	56%	16%	42%	1.8+/-0.1

Ethnicity	Number	Cashew	Pepper	Rice	Cassava	Coffee
Kinh	146	*71%*[1]	**45%**	*9%*	19%	**20%**[2]
S'tiếng	247	**84%**	*11%*	**63%**	*17%*	*1%*
Tày	29	83%	31%	**66%**[2]	**59%**	10%
Nùng	36	83%	**61%**	**86%**	**92%**	*3%*[1]
Hoa	24	*48%*	40%	*0*	*0*	**100%**
Mixed	44	77%	39%	48%	25%	25%
Other	5	60%	20%	20%	20%	80%
Total	531	78%	28%	45%	25%	14%

Tables 6.25. Economic indicators by ethnic group. Cell contents are percentage of households responding in the affirmative to questions of item ownership, crop cultivation, having sufficient money or any months with insufficient rice for the household's needs, except mean months (+/- SE) without rice. (Bold denotes significantly more than average, italic significantly less. * - significantly more than Kinh (but not than the average) [+]- significantly less than Kinh). All p values <0.005 except 1) 0.05>=p>=0.03 2) p=0.01.

Occupation and Education

Unsurprisingly, 98% of economically active adult study subjects were identified as farmers. All 35 individuals not farming (24 traders, and a few teachers, policemen, mechanics and rubber workers) were all Kinh, although the vast majority of Kinh were farmers. It is probable that this survey slightly underestimated the number of administrative or professional households in the 3 communes, as these families are more likely to have been selected out by the interviewing teams, in order not to cause offence.

Forty percent of study subjects had no formal education, 34% had completed the 1st grade, 20% the second, and only 6% 3rd or higher. There were significant differences in educational attainment between different ethnic groups (table 6.26). The Kinh were by far the best educated, followed by the Tày. Very few S'tiếng, Nùng or Hoa had progressed beyond the second grade. A very high proportion of the S'tiếng had received no formal education at all, although fortunately this was less common in the younger age groups (table 6.27), a trend which was not seen in the other ethnic groups.

Ethnicity	No schooling	First grade	Second grade	Third grade	University	Total
Kinh	***6 (3%)***	73 (32%)	**106 (47%)**	**35 (16%)**	**5 (2%)**[1]	225
S'tiếng	**250 (64%)**	***117 (30%)***[1]	***23 (6%)***	***2 (1%)***	***0***[1]	392
Tày	***4 (9%)***	19 (44%)	14 (33%)	**6 (14%)**[2]	0	43
Nùng	15 (28%)	23 (43%)	14 (26%)	2 (4%)	0	54
Hoa	11 (28%)	**21 (53%)**[2]	8 (20%)	0	0	40
Other	***1 (9%)***[2]	**9 (82%)**	0	0	1 (9%)	11
Total	287 (37%)	262 (34%)	166 (22%)	45 (6%)	6 (1%)	766

Table 6.26. Educational attainment by ethnic group. Cell contents are number (percentage) of ethnic group achieving educational level. Bold font indicates significantly greater, and bold italic font significantly smaller proportion than other ethnic groups. All p values <0.005 except 1) p=0.01 2) 0.03<=p<=0.05.

Age Group	No schooling	1st grade	2nd grade	3rd grade	Total
15-24	21 (27%)	41 (53%)	13 (17%)	2 (3%)	77
25-34	69 (66%)	33 (31%)	3 (3%)	0	105
35-44	63 (72%)	24 (27%)	1 (1%)	0	88
45-54	43 (83%)	8 (15%)	1 (2%)	0	52
55-64	27 (87%)	4 (13%)	0	0	31
65+	21 (91%)	1 (4%)	1 (4%)	0	23
Total	244 (65%)	111 (30%)	19 (5%)	2 (1%)	376

Table 6.27. Educational attainment by age group amongst the S'tiếng.

Respondents knowledge of malaria was worse than expected: 51% professing ignorance of the mode of transmission of malaria. Of the 419 who purported to know, 316 gave explicit answers, of which only 2 were completely wrong (not mentioning mosquitoes), and a further 18 included incorrect modes of transmission (mostly in connection with drinking water) as well as naming mosquitoes as the culprit. The highest proportions of knowledgeable individuals were amongst the Kinh and Hoa (75 and 80% respectively), although none of the Hoa interviewees gave details compared with 86% of the Kinh.

42% of the Tày, 34% of S'tiếng and only 22% of the Nùng respondents reported knowing how malaria was transmitted, with 61%, 83%, and 100% of those individuals giving a detailed response respectively. There was no difference between ethnic groups in the proportion of wrong or spurious modes of transmission amongst those giving detailed answers. There were also significant differences between ethnic groups in the proportion believing that malaria is preventable, and in those understanding that antimalarials are free (table 6.28). There was almost complete concordance between knowing how malaria was transmitted and believing that malaria is preventable, with single figure percentages in the discordant cells with two interesting exceptions: 17% of Kinh respondents who professed not to know how malaria is transmitted still thought it was preventable, and 16% of the S'tiếng interviewees who knew how malaria was transmitted still believed it was not preventable. The concordance with knowing antimalarials were free was weaker but remained symmetrical both overall and within each ethnic group, with the exception of the Hoa, very few of whom knew that antimalarials were free (tables 42-44, appendix 2). There was a marked correlation between educational achievement and malaria knowledge, which held across all ethnic groups except the Tày (table 6.29). Multivariate analysis suggested the educational effect was dominant over the effects of ethnicity.

Ethnic group	Know how malaria is transmitted	Believe malaria is preventable	Believe antimalarials are free
Kinh	**170 (75%)**	**175 (77%)**	**137 (65%)**
S'tiếng	***126 (34%)***	***99 (30%)***	121 (45%)
Tày	18 (42%)	19 (45%)	19 (46%)
Nùng	***12 (23%)***	***11 (21%)***	***11 (21%)***
Hoa	**32 (80%)**	**33 (83%)**	***5 (13%)***
Other	**11 (100%)**	**11 (100%)**	***0***[1]
Total	370 (50%)	349 (49%)	294 (47%)

Table 6.28. Variation in antimalarial knowledge. Cell contents are number giving an affirmative response (percentage of those supplying valid answer). Bold font indicates a significantly greater, and bold italic font significantly smaller, proportion than other ethnic groups. All p values <0.001 except 1) 0.007.

Education	Kinh	S'tiêng	Tày	Nùng	Hoa	Total
No schooling	3 (50%)	58 (25%)	3 (75%)	1 (7%)	8 (73%)	87 (27%)
1st grade	48 (68%)	51 (45%)	6 (32%)	4 (17%)	17 (81%)	161 (56%)
2nd grade	76 (72%)	14 (61%)	6 (43%)	5 (38%)	7 (88%)	116 (66%)
3rd grade	34 (100%)	2 (100%)	3 (50%)	2 (100%)	0	44 (94%)
University	5 (100%)	0	0	0	0	6 (100%)
Total	166 (75%)	125 (34%)	18 (42%)	12 (23%)	32 (80%)	414 (49%)

Table 6.29. Variation in number (percentage) of individuals professing to know how malaria is transmitted with educational attainment and ethnic group.

Malaria associations

Blood smears were taken in hamlets in Đức Hạnh and Đa Kia only. The overall prevalence of smear positivity in this survey was 3.4% (table 6.30). Thirty of the 867 slides were not sufficiently well labelled to allow identification of the individual bled. Four of these 30 slides were positive, leaving an overall smear positive prevalence of 3.1% amongst the survey subjects. The hamlet, ethnic and age group breakdown are given in tables 6.30, 6.31, and 6.32. Demonstrating any association between behaviour and malaria in the face of such a low event rate is difficult. The problem is compounded by differences in age profile of ethnic group subsamples (table 6.33), ethnic minority groups have higher proportions of younger age groups (numerically dominated by the S'tiêng), whilst the Kinh subjects are concentrated in middle age. Thus even if any significant associations were found, correcting for age and ethnic group would be almost certain to abolish the significance. On the premise that a skew of 20 to 4 or greater might be a reasonably convincing result, however, the primary outcome variables were tested against smear status, and the results presented below. Having accumulated 3 years of smear data using the same sampling frame as the KAP gave us an opportunity to test current behaviour against objectively documented malaria status. There are a number of problems with this approach, which have been discussed in Chapter 2. The three malaria indices eventually chosen (for both individuals and households) were: smear status in KAP (binary, houses positive if any individual positive), smear positive in any survey (again houses were positive if any member in any survey was positive) and a continuous adjusted smear status

index, which attempted to correct for incomplete sampling in the face of a secular trend towards decreasing malaria prevalence (details in Methods-Analysis, Chapter 2). Very few significant differences were seen in this third index, and as it is difficult to tabulate, most association tables detail only the first two, labelled "KAP" and "All" respectively.

Hamlet	Number of slides	Smear positive	*P. vivax*	*P. falciparum*
Bù Gia Phúc 1	84	3 (4%)	3	0
Bù Gia Phúc 2	71	5 (7%)	1	4
Đak Khâu	185	1 (1%)	0	1
Thác Dài	163	9 (6%)	4	5
Bình Giải	166	5 (3%)	3	1
Bù Tâm	168	3 (2%)	2	1
Total	837	26 (3%)	13	12

Table 6.30. Smear positive prevalence by hamlet. No significant differences.

Ethnic group	Number of slides	Smear positive	p value
Kinh	170	2%	0.27
S'tiếng	364	4%	0.38
Tày	34	0	0.29
Nùng	65	5%	0.43
Hoa	48	4%	0.63
Other	13	0	
Total	694	3%	

Table 6.31. Smear positive prevalence by ethnic group. No significant differences.

Age group	Number of slides	Smear positive
<2	16	0
2-4	62	7%
5-9	89	8%
10-14	51	2%
15-24	92	2%
25-34	181	3%
35-44	162	3%
45-54	106	0
55-64	42	2%
65+	36	3%
Total	837	3%

Table 6.32. Smear positive prevalence by age group. Regression of smear positivity on age significant p=0.03.

Age group	Kinh	S'tiếng	Tày	Nùng	Hoa	Other	Total
<2	2%	2%	0	2%	2%	8%	14
2-4	4%	7%	15%	12%	10%	8%	49
5-9	5%	14%	15%	12%	8%	8%	77
10-14	7%	7%	6%	9%	6%	0	48
15-24	12%	11%	6%	5%	15%	15%	75
25-34	25%	17%	29%	34%	19%	31%	151
35-44	22%	17%	21%	15%	19%	31%	129
45-54	20%	11%	9%	5%	15%	0	88
55-64	3%	8%	0	3%	4%	0	37
65+	1%	6%	0	3%	2%	0	26
Total	170	364	34	65	48	13	694

Table 6.33. Age and ethnic group breakdown of those individuals bled for blood film. Cell contents are percentage of ethnic subsample in specified age group.

The possibility of behaviour changing in the years between the malariometric survey and the KAP is essentially impossible to correct for, as recall bias would render invalid any retrospective questions over that time scale. It is, however, reasonable to consider which, if any, behaviours are likely to have changed over a mere three years. Switching cultivation practices or acquiring livestock takes time, and changes over this period are likely to be small. The chances of rebuilding one's house in different materials over the course of 3 years are also slim. Whilst annual income and ownership of a television or motorbike might well have changed in this time, social and income mobility is limited in this setting, and relative differences are likely, for the most part, to be preserved. This is not universally true, however, and some households will have improved their relative socioeconomic status, whilst that of others will have declined. Devastating events such as the loss of a family breadwinner are, fortunately, rare. Whether forest and field sleeping behaviour would have altered during our period of surveillance is difficult to establish. On the one hand, policies of Vietnamisation have pushed ethnic minorities into more sedentary cultivation methods, on the other, legal and illegal acquisition of land which minority groups regard as theirs has, in many cases, pushed them into increasingly remote areas. Increased forest destruction, predominantly cleared for agricultural use by incoming migrants, may have pushed the forest edge beyond a reasonable travelling distance, but, by the same token, this might have increased the likelihood that individuals visiting the forest would sleep there. It seems improbable, but not impossible, that these forces would have induced significant change in just 3 years. Bed net use is also very much a matter of habit, but bed net ownership is not. There have been a number of episodes of bednet distribution in the district in the last three years, which may well have increased the proportion of individuals protected (although no consistent trend was seen in the data gathered on bednet use in the cross sectional surveys). Knowledge and understanding of malaria can also change over this time period, although there have been no large scale education campaigns undertaken in the district during the study period or the antecedent year.

Demographic factors and malaria

Ethnic group and age were inevitably associated with retrospective indices of malaria, although there was no association with any aspect of household age structure. Smear positivity in this survey was not associated with ethnic group (table 6.31), although once again a significant age effect was apparent at the individual level regardless of whether a cut off of 15 was used (5.5% vs 2.3% p=0.019) or 2 to 9 year olds were compared with other age groups (7.3% vs 2.2% p=0.001). Full age group breakdown can be found in table 46, appendix 2. Hamlet and village were not associated with smear prevalence in this survey (table 47, appendix 2), but there were, obviously, significant variations of the retrospective indices with both. The same was true of migrant status (3% smear positive in both categories in this survey).

Bednet use and malaria

Bed net use in the home was significantly protective against smear positivity in this survey (table 6.34), albeit on exceptionally small numbers. There was clearly some discrepancy between bednet use reported at the household and individual level, as none of the ten households reporting not using bednets had a single smear positive member. Current bed net use did not significantly affect any of the retrospective malaria indices at individual or household level. Most measures of bednet quantity and quality (total numbers of bednets, bednets per individual, bednets per adult, good quality bednets, good quality bednets per adult and proportion of household bednets deemed to be in good condition) were not associated with protection from current or previous malaria (table 48, appendix 2). The exception was how bed nets had been treated: households and individuals in those households had less malaria if the bednets had been treated, and there was a trend to decreasing smear prevalence with decreasing interval since last treatment (tables 6.35, 6.36). Once again the numbers are very small, however, and smear indices

from previous surveys appear to show a trend in the opposite direction, casting more than a little doubt on the validity of this association.

Bednet use in field or forest was not protective (table 6.37).

	Survey	Bednet users	Bednet nonusers	p
Individual	KAP	3% (23/807)	10% (3/30)	0.03
	All	11% (157/1480)	7% (3/41)	0.50
Household	KAP	6% (24/383)	0% (0/10)	0.41
	All	23% (107/472)	0% (0/10)	0.09

Table 6.34. Smear prevalence in users and nonusers of bednets at home in this survey and all surveys.

	Survey	Bednets treated	Bednets untreated	p
Individual	KAP	2% (16/661)	7% (10/145)	0.006
	All	11% (142/1292)	9% (16/185)	0.34
Household	KAP	3% (23/807)	10% (3/30)	0.03
	All	11% (157/1480)	7% (3/38)	0.50

Table 6.35. Bed net insecticide treatment and smear positivity in this survey (KAP) and all surveys.

Bednet last treated	Percentage of households smear positive (number/total)	
	KAP	All
Never	12% (10/82)	17% (15/88)
Over 1 year ago	0% (0/39)	8% (4/52)
6-12 months ago	7% (13/188)	29% (76/264)
Within the last 6 months	2% (1/56)	24% (14/58)
Total	7% (24/370)	24% (109/462)
p value	0.05	0.02

Table 6.36. Bednet treatment and smear positivity. p values given by non-parametric test for trend.

Survey	Users of bednets in field or forest	Non-users of bednets in field or forest	p
KAP	5% (8/149)	3% (7/221)	0.29
All	10% (28/271)	7% (18/273)	0.12

Table 6.37. Percentage of individuals smear positive by bednet use in field or forest and survey.

	Kinh	S'tiêng	Tày	Nùng	Total
Only free nets	10% (10)	2% (52)	0 (9)	43% (7)	6% (78)
Bought & free nets	6% (18)	13% (38)	0 (8)	15% (13)	10% (77)
Only bought nets	22% (9)	0 (14)	0 (1)	20% (5)	10% (29)

Table 6.38. Percentage smear positive in previous surveys by procurement of bed nets, by ethnic group. Cell contents are percentage smear positive (total number of observations in category).

Bednet ownership was highlighted as one of the variables that might well have altered during the 3 years of malaria surveillance. If this were to have been the case, one might expect to find a higher past prevalence of malaria in households relying on freely distributed nets, particularly in some ethnic groups. With the exception of the Nùng, such a phenomenon is not apparent (table 6.38), although numbers are small.

Field and forest activity and malaria

Problems in the interpretation of individuals sleeping in the field were discussed above. Given these difficulties, it is perhaps not surprising, therefore, that no association is apparent between individual or household risk of malaria and whether or not an individual or any member of a household is reported to sleep in the field. We might also be sceptical of the finding that household malaria risk in this survey increases with the percentage of individuals sleeping in the field (ethnically adjusted mean+/-SE: 28%+/-1.5 amongst smear negative households vs 44%+/-7.7% amongst smear positive households, p=0.04). There was no such association with previous binary malaria risk, but there was a weak correlation between the proportion of field sleepers and the adjusted household malaria index (fig 10 appendix 2). Sleeping in a plot hut rather than a house, however, did significantly increase malaria prevalence in this and previous surveys (table 6.39), and houses which had experienced malaria had a higher proportion of farmers (0.91 vs 0.85, p=0.02), suggesting that field activity and malaria risk were not unrelated. An intriguing association in this survey was a significant difference in the journey time to a household's fields between smear positive and negative households (57 vs 25 mins, p=0.003), although this did not hold for retrospective smear indices (34 vs 26 mins, p=0.223).

Survey	Plot hut	House	p
KAP	12% (8/69)	1% (1/91)	0.004
All	14% (13/96)	1% (1/98)	0.001

Table 6.39. Smear positive percentages amongst individuals sleeping in the field who slept in houses or plot huts.

House construction and malaria

House construction had less effect on malaria status than expected (tables 6.40). Whilst smear positivity in this survey appears to be more prevalent in bamboo walled houses and those with thatched roofs, neither association reaches significance, and neither is associated with malaria prevalence in previous surveys. The significant association between tiled roofs and retrospective malaria indices disappears on adjustment for ethnic group, as most households utilising this material are S'tiếng or Nùng (table 6.14). The general "openness" index of the house didn't seem to affect malaria, and we were surprised to see no protective effects from brick construction or glass windows. Wooden shutters on the windows did reduce risk, but only in this survey (table 6.41). The distance to the nearest stream was not associated with malaria prevalence, whether analysed as a continuous variable or across previously cited breakpoints at 250 and 500m (table 49, appendix 2). Houses which had been documented to contain smear positive individuals in previous surveys tended to be further from animals pens than those which had not (37m vs 11m, p=0.04), but this association did not hold in this survey (9 vs 19m, p=0.78), although there were only 9 smear positive households for which this information had been recorded. The distance to the nearest road was our best remoteness indicator, and showed no association with any household indicator of malaria (table 50 appendix 2).

	Wall				Roof			
Survey	KAP		All		KAP		All	
Material	N	Smear positive	N	Smear positive	N	Smear positive	N	Smear positive
Metal sheeting/ corrugated iron	4	0	4	0	237	5%	311	19%
Wattle/ Thatch	9	11%	9	33%	95	9%[2]	106	23%
Bamboo	155	8%[1]	183	20%	0	0	0	0
Wood	188	3%	248	23%	4	0	4	50%
Brick	28	4%	35	23%	0	0	0	0
Tile	0	0	0	0	55	4%	65	32%[3]
Other	8	13%	10	20%	2	0	4	0
Total	392	6%	489	22%	393	6%	490	22%

Table 6.40. Construction materials and risk of smear positivity. Notes:
1) p=0.054 vs all other materials; 2) p=0.07; 3) p=0.036, but see text.

Survey	KAP		All	
Material	N	Smear positive	N	Smear positive
Open windows	106	7%	112	23%
Bamboo shutters	27	11%	30	30%
Wooden shutters	123	2%[1]	169	21%
Glass windows	8	0	9	33%
No windows	116	6%	151	17%
Total	380	5%	471	21%

Table 6.41. Window materials and risk of positive individual in household. (1:p=0.04)

Economic status and malaria

There was no significant relationship between malaria and any measure of household income (table 6.42). The majority of smear positive individuals in this survey came from houses in the lowest income quartile, but this was followed by the highest quartile (table 6.43). No trend at all was apparent in the retrospective malaria indices. Adjusting for ethnic group resulted in a trend towards a positive association between income and all surveys malaria, which did not begin to reach significance, and had no effect on the relationship in this survey (tables 6.44, 6.45). Surrogate markers of household wealth such as motorbike or TV ownership were not associated with malaria, either alone or adjusted for ethnic group (table 6.46). Somewhat surprisingly, ownership of a bicycle or a radio (low value items) was associated with a trend to lower prevalence of malaria, which reached significance for bicycle ownership and all surveys smear status (table 6.46). This relationship held across ethnic groups. There was no relationship between smear status and other surrogate markers of wealth such as number of rooms in the house (both unadjusted and adjusted for number of adults inhabitants), area of land owned, whether there were

Survey	Smear negative	Smear positive	p
KAP	12.1+/-0.8	11.4+/-4.1	0.84
All surveys	12.6+/-0.8	10.7+/-1.0	0.24

Table 6.42: Annual income (million VNĐ) by smear status in KAP and all surveys (Mean +/- SE)

Income quartile	KAP	All surveys
Lowest	**11% (9/84)**	24% (25/105)
3rd	2% (2/110)	23% (33/143)
2nd	0 (0/44)	16% (9/55)
Highest	6% (4/70)	20% (19/96)
Total	5% (15/308)	22% (86/399)

Table 6.43: Smear status and income quartile. Bold type indicates p=0.001 vs all others.

any months in which the family did not have enough food, the number of months with insufficient food and the subjective assessment of whether the household earned enough money to fulfil its needs or not. The last three did show non-significant trends in the direction we might have expected, but the others demonstrated either no pattern, or conflicting non-significant patterns in this and previous surveys.

	KAP smear status			All surveys smear status		
Ethnicity	Negative	Positive	p	Negative	Positive	p
Kinh	17.7+/-2.2	22.3+/-9.6	0.66	18.1+/-2.0	14.7+/-4.7	0.61
S'tiếng	8.5+/-0.8	2.7+/-1.0	0.09	7.8+/-0.8	9.6+/-1.2	0.20
Tày				13.4+/-2.5	9.8+/-3.9	0.53
Nùng	20.2+/-6.1	40.0+/-10.0	0.36	10.0+/-1.4	11.7+/-3.8	0.64
Hoa				20.2+/-6.1	40.0+/-10.0	0.36
Mixed				16.1+/-3.7	15.0+/-3.6	0.90

Table 6.44. Mean annual income (million VNĐ) and smear status in this and all surveys by ethnic group. Mean+/-SE.

	KAP			
Income quartile	Kinh	S'tiếng	Nùng	Hoa
Lowest	0 (0/6)	10% (5/48)	20% (1/5)	0 (0/1)
3rd	5% (1/20)	3% (1/39)	0 (0/14)	0 (0/10)
2nd	0 (0/13)	0 (0/9)	0 (0/4)	0 (0/6)
Highest	10% (2/21)	0 (0/16)	0 (0/4)	25% (2/8)
Total	5% (3/60)	5% (6/112)	4% (1/27)	8% (2/25)

	All surveys				
Income quartile	Kinh	S'tiếng	Tày	Nùng	Hoa
Lowest	13% (1/8)	22% (14/65)	20% (1/5)	40% (2/5)	0 (0/1)
3rd	8% (2/24)	39% (22/57)	25% (2/8)	11% (2/18)	0 (0/10)
2nd	12% (2/17)	31% (4/13)	0 (0/2)	0 (0/5)	0 (0/6)
Highest	7% (2/27)	38% (9/24)	14% (1/7)	33% (2/6)	25% (2/8)
Total	9% (7/76)	31% (49/159)	18% (4/22)	18% (6/34)	8% (2/25)

Tables 6.45. Percentage (number/total) smear positive by income quartile. No significant trends.

The relationship between crop choice or livestock ownership and household income was discussed above. These variables were thus investigated as potential confounders. Ownership of either cattle or pigs was protective against malaria in this survey (table 6.47). There were no ethnic differences in this relationship, although the small number of events results in a loss of significance in any ethnic group considered separately. There was

absolutely no effect, however, on past malaria risk (table 6.47), once again casting doubt on the association. With the exception of pepper, the opposite was true for crop choices. Cashew and rice cultivation were associated with increased past malaria risk, and coffee with reduced risk, but none had any effect on household smear positivity in this survey. The relationship in previous surveys did not survive correction for ethnic group, and some of the effects appeared to operate in different directions in households of different ethnicities (table 51, appendix 2). Pepper cultivation was consistently associated with a lower risk of malaria (table 6.48).

	Survey	KAP					All Surveys				
		Households not owning item		Households owning item		p	Households not owning item		Households owning item		p
Item	Ethnic group	N	Percent positive	N	Percent positive		N	Percent positive	N	Percent positive	
Honda	Kinh	11	9%	70	4%	0.494	13	8%	84	10%	0.832
	S'tiếng	48	8%	120	8%	0.855	64	22%	156	35%	0.052
	Tày	5	0	8	0	N/A	6	33%	16	13%	0.259
	Nùng	12	8%	16	6%	0.832	13	15%	22	23%	0.6
	Hoa	4	0	21	10%	0.52	4	0	21	10%	0.52
	Total	103	7%	294	6%	0.71	190	7%	207	5%	0.289
TV	Kinh	24	4%	57	5%	0.835	27	7%	70	10%	0.693
	S'tiếng	95	11%	73	4%	0.123	122	24%	98	41%	0.007
	Tày	5	0	8	0	N/A	7	14%	15	20%	0.746
	Nùng	12	8%	16	6%	0.832	15	20%	20	20%	1.0
	Hoa	5	0	20	10%	0.461	5	0	20	10%	0.461
	Total	264	7%	133	4%	0.175	326	7%	71	3%	0.208
Bike	Kinh	59	5%	22	5%	0.921	75	11%	22	5%	0.384
	S'tiếng	113	10%	55	4%	0.165	163	35%	57	21%	0.051
	Tày	5	0	8	0	N/A	14	29%	8	0	0.095
	Nùng	4	25%	24	4%	0.134	11	27%	24	17%	0.466
	Hoa	22	5%	3	33%	0.085	22	5%	3	33%	0.085
	Total	125	18%	369	24%	0.229	230	21%	264	23%	0.486
Radio	Kinh	56	4%	25	8%	0.395	71	10%	26	8%	0.745
	S'tiếng	150	9%	18	0	0.193	194	31%	26	35%	0.704
	Tày	10	0	3	0	N/A	19	21%	3	0	0.38
	Nùng	13	15%	15	0	0.115	20	25%	15	13%	0.393
	Hoa	24	8%	1	0	0.763	24	8%	1	0	0.763
	Total	359	25%	135	15%	0.015	414	23%	80	18%	0.263

Table 6.46. Ethnic variation in ownership of key household items.

	Survey	KAP					All Surveys				
		Households not owning animal		Households owning animal		p	Households not owning animal		Households owning animal		p
Animal	Ethnic group	N	Percent positive	N	Percent positive		N	Percent positive	N	Percent positive	
Cows	Kinh	70	6%	11	0	0.42	84	11%	13	0	0.22
	S'tiêng	144	9%	24	0	0.13	184	30%	36	39%	0.29
	Tày	11	0	2	0	N/A	20	20%	2	0	0.48
	Nùng	13	15%	15	0	0.12	**20**	**35%**	**15**	**0**	**0.01**
	Hoa	25	8%	0	N/A	N/A	25	8%	0	N/A	N/A
	Total	**331**	**7%**	**66**	**0**	**0.02**	413	22%	81	25%	0.57
Pigs	Kinh	70	6%	11	0	0.42	80	11%	17	0	0.15
	S'tiêng	124	10%	44	2%	0.11	165	32%	55	31%	0.93
	Tày	11	0	2	0	N/A	19	21%	3	0	0.38
	Nùng	24	8%	4	0	0.55	29	24%	6	0	0.18
	Hoa	24	8%	1	0	0.76	24	8%	1	0	0.76
	Total	**314**	**7%**	**83**	**1%**	**0.04**	389	22%	105	22%	0.92

Table 6.47. Livestock ownership and smear positivity, by ethnic group

Crop	Survey	Not growing crop		Growing crop		p
		N	Percent positive	N	Percent positive	
Cashew	KAP	99	6%	298	6%	0.99
	All surveys	105	13%	389	25%	0.01
Pepper	KAP	321	7%	76	1%	0.05
	All surveys	381	26%	113	10%	<0.001
Rice	KAP	255	6%	142	6%	0.80
	All surveys	283	16%	211	31%	<0.001
Cassava	KAP	293	7%	104	3%	0.12
	All surveys	365	23%	129	19%	0.36
Coffee	KAP	335	6%	62	5%	0.66
	All surveys	418	25%	76	8%	0.001

Table 6.48. Crop choice and malaria

Occupation, education and malaria

The effect of the proportion of farmers in a household has been discussed above. Being a farmer was also a risk factor at an individual level in previous surveys (table 6.49), but not this one. The number of individuals occupied in specific trades or professions other than farming was too small to allow further analysis: in particular we only captured one individual with a forest related trade. Cropping patterns already described could be examined from an occupational viewpoint: pepper is usually grown close to the house, cashews usually at some distance, and coffee, corn or cassava may be either close or

distant. There were only 10 families growing pepper without one of the other listed crops, insufficient to examine their malaria risk separately.

Survey	Farmers	Non-farmers	p
KAP	3% (13/531)	1% (1/78)	0.52
All	8% (67/836)	2% (2/101)	0.03

Table 6.49. Risk of smear positivity by occupation as farmer

Education did not protect against malaria at the individual level (table 6.50). There were insufficient observations to stratify by ethnic group in this survey, but malaria prevalence in all surveys seemed to increase with increasing education in most ethnic groups (table 6.50). None of these trends was remotely significant, and might represent a falling denominator with very few events in each stratum. Dichotomous analysis of those with any education, or those with secondary education, against those without, suggested the same trend. The only exception was amongst the S'tiếng, where literacy (in Vietnamese – S'tiếng is a purely oral language) was non-significantly associated with protection from malaria (table 6.51). There were too few illiterate members of the other ethnic groups to check the validity of this trend outside the S'tiếng. Paradoxically, there was an effect of education at the household level, although not in this survey. Those households with at least one member educated to secondary school level or higher had a diminished risk of ever having contained a smear positive individual (table 6.52).

Education	Kinh	S'tiếng	Nùng	All surveys total	KAP total
No schooling	0 (4)	6% (207)	8% (12)	6% (235)	3% (263)
1st grade	2% (62)	8% (79)	17% (23)	5% (200)	3% (194)
2nd grade	4% (57)	8% (12)	17% (12)	5% (96)	2% (94)
3rd grade	10% (21)	0 (2)	0 (1)	6% (29)	4% (27)
University	0 (4)	(0)	(0)	0 (4)	0 (4)
Total	3% (147)	7% (300)	15% (48)	6% (544)	3% (582)

Table 6.50. Percentage smear positive (number of observations) by education level. Ethnic breakdown for all surveys smear result only. No Hoa or Tày individuals for whom we have educational attainment data were smear positive. No significant differences.

	All surveys	KAP
Illiterate	9.3% (108)	5.9% (102)
Literate	5.6% (180)	1.6% (129)
p value	0.231	0.074
Total	6.9% (288)	3.5% (231)

Table 6.51. Smear positive prevalence (total sample) amongst literate and illiterate S'tiếng.

Survey	Houses with any educated member	Houses without any educated member	p
KAP	6% (13/237)	7% (11/155)	0.52
All	21% (66/312)	25% (44/177)	0.35

Survey	Houses with any secondary school graduate	Houses without a secondary school graduate	P
KAP	4% (4/101)	7% (20/291)	0.29
All	16% (21/132)	25% (89/357)	0.03

Table 6.52. Percentage of smear positive households by highest educational attainment of any household member.

Health care seeking behaviour and malaria

Families choosing the health station as their primary health care provider appeared to be at increased risk of having harboured a smear positive individual at some time (table 6.53). This effect remained significant after adjustment for ethnic group and village. The same trend was apparent in this survey, and reached significance for the question of where a family would seek help with a sick child. The relationship between malaria and the provider of the last course of treatment administered to children and adults in the family was consistent with these results, although the risk was only significant for children, and only with regard to malaria episodes in all surveys (table 6.54). The direction of the effect was consistent across ethnic groups and villages, but adjusting for either abolished the statistical significance of the association (tables 52-54, appendix 2). There was no association at the individual level between paying for antimalarials and smear positivity in this or previous surveys, or between knowing that antimalarials were provided free of charge and any malaria index. Amalgamated individual data on the healthcare provider responsible for treating the last malaria episode in the house also showed a positive

association between family malaria risk across all surveys and having been treated at the health station. This effect was consistent across communes and ethnic groups (table 54, appendix 2), but was not seen in this survey (table 6.55). Whilst the different indicators of health care seeking behaviour at the household level give consistent results, they conflict absolutely with the associations found with individually recounted treatment patterns. There is no effect of choosing the health station as provider in this data set, but significant risk attached to self treatment (table 6.56). This result was also consistent across age and ethnic groups (tables 55 & 56, appendix 2), and between this survey and all surveys, although the small number of individually interviewed family members with previous smears meant this survey dominated all surveys in the individual dataset. Possible reasons for this discrepancy are discussed below.

There appeared to be no effect on malaria risk whatsoever of professing to know how malaria is transmitted or knowing that antimalarials were free. This was true in all ethnic groups (tables 57 & 58, appendix 2).

	Survey	KAP	p	All surveys	p
First choice healthcare provider if adult ill	Health station	10% (70)	0.08	34% (107)	0.001
	Hospital	2% (91)	0.11	18% (92)	0.35
	Private doctor	6% (137)	0.89	17% (138)	0.07
	Depends on illness	5% (73)	0.95	22% (130)	1.0
	Total	6% (391)		22% (488)	
First choice healthcare provider if child ill	Health station	13% (72)	0.01	37% (112)	<0.001
	Hospital	2% (87)	0.10	18% (88)	0.29
	Private doctor	6% (125)	0.80	16% (126)	0.04
	Depends on illness	4% (70)	0.51	21% (122)	0.73
	Total	6% (369		22% (464)	

Table 6.53. Choice of health care provider and risk of malaria. Cell contents are percentage of households smear positive (total number choosing that healthcare provider). p values are for the healthcare provider vs all others.

	Survey	KAP	p	All surveys	p
Last time adult in house was unwell medicine obtained from:	Market	2% (81)	0.32	20% (121)	0.49
	Private doctor	5% (151)	0.47	23% (160)	0.85
	Health station (bought)	8% (40)	0.31	25% (75)	0.44
	Health station (free)	0 (16)	0.38	33% (24)	0.17
	Hospital	4% (28)	0.82	10% (29)	0.12
	Total	4% (316)		22% (409)	
Last time child in house was unwell medicine obtained from:	Market	3% (64)	0.29	18% (92)	0.30
	Private doctor	6% (113)	0.80	18% (120)	0.19
	Health station (bought)	10% (30)	0.29	33% (64)	0.03
	Health station (free)	10% (20)	0.40	37% (35)	0.03
	Hospital	0 (15)	0.32	0 (16)	0.03
	Total	6% (242)		22% (327)	

Table 6.54. Source of most recent medicine taken by adult or child in household and risk of malaria. Cell contents as table 6.53.

	Survey	KAP	p	All surveys	p
Healthcare provider chosen for last episode of malaria	Health station	7% (42)	0.78	34% (74)	0.02
	Hospital	6% (54)	0.84	16% (57)	0.13
	Private office	4% (110)	0.17	19% (113)	0.20
	Other/not treated	14% (7)	0.37	38% (8)	0.35
	Self treat	2% (53)	0.16	17% (60)	0.16
	Health volunteer	0 (1)	0.80	0 (1)	0.58
	Varied within household	14% (57)	0.01	33% (58)	0.08
	Total	6% (324)		24% (371)	

Table 6.55. Healthcare provider chosen for last episode of malaria. Information aggregated at household level from individual questionnaires. Cell contents as table 6.53.

	Survey	KAP	p	All surveys	p
Healthcare provider chosen for last episode of malaria	Health station	2% (58)	0.54	6% (88)	0.82
	Hospital	3% (86)	0.78	3% (91)	0.20
	Private office	2% (173)	0.48	8% (183)	0.30
	Other/no treatment	21% (14)	<0.001	18% (17)	0.05
	Self treat	2% (95)	0.56	5% (100)	0.58
	Health volunteer	0 (5)	0.69	0 (5)	0.56
	Total	3% (431)		6% (484)	0.82
Action taken if became unwell whilst working in fields	Self treat at home	16% (32)	0.001	17% (35)	0.01
	Self treat in field	0 (2)	0.76	0 (2)	0.70
	Go to health station	2% (47)	0.35	5% (74)	0.49
	Go to private doctor	3% (64)	0.49	7% (69)	0.95
	Go to hospital	0 (31)	0.18	0 (32)	0.09
	Total	5% (176)		7% (212)	

Table 6.56. Healthcare provider chosen for last episode of malaria, action taken if unwell in field, and malaria risk at the individual level. Cell contents percentage of individuals smear positive (total number of individuals choosing that healthcare provider). p values are for that healthcare provider vs all others.

Discussion

The primary purpose of this study was to test the hypothesis that social and behavioural risk factors could explain the difference in malaria prevalence between the S'tiếng and other ethnic groups, in particular the majority Kinh. Table 6.57 gives a qualitative hierarchy of ethnic group risk in the major categories assessed. This ranking is based on results from other studies which have suggested or established that sleeping in field or forest, use of bamboo or thatch in house construction, greater proximity to streams, use of self treatment rather than formal health services, poor knowledge of malaria transmission, and poverty are associated with increased malaria risk, and that use of bednets at home and in the field, keeping nets well maintained and treated, owning animals, better malaria knowledge and greater general education are protective. The hierarchy is necessarily qualitative, as performing quantitative analysis on aggregated fields would require assignment of weight to different risk factors, which is not justified by previous data. Even qualitative combination of all categories into a single ranking would be fraught with such difficulties.

Risk factor category	Ethnic group risk order
Bednet ownership, use and care	Nùng > S'tiếng > Hoa > Tày = Kinh
Field and forest activity	Hoa > Tày >= S'tiếng > Nùng > Kinh
House construction and location	Tày > S'tiếng > Nùng = Hoa > Kinh
Occupation and farming methods (order least clear in this category)	Nùng = S'tiếng = Tày = Hoa > Kinh
Malaria knowledge and healthcare seeking behaviour	Nùng > S'tiếng = Tày > Kinh >= Hoa
Socioeconomic status	S'tiếng >= Nùng = Tày > Kinh >= Hoa

Table 6.57. Qualitative ranking of malaria risk by behavioural or environmental risk factor category.

The consistently relatively low risk behaviour of the majority Kinh is evident from the table. Very few studies of ethnic variation in malaria risk behaviour have been published in Medline indexed journals. There are a number of possible reasons why this should be so. Most KAP studies are undertaken as part of malaria control, rather than malaria research, programmes. Most results are therefore contained in project reports, rather than

medical or sociological journals, and are presented with implementation goals, rather than academic interest, in mind. In particular, comparative studies are rather infrequent, most projects focusing on the knowledge and practices of a particular, pre-assigned group (be it geographically or ethnically defined). Few statistically significant differences have been found in the small number of published comparative studies, which may reflect the small numbers of individuals from any one ethnic group included in most samples (almost inevitable in any ethnically diverse environment), or the relatively small differences that actually exist in any one behavioural trait. The question of whether these small differences are clinically significant by themselves (a relatively modest increase in annual income might make a big difference in "disposable" income available for healthcare, or a few percentage points difference in bed net use might take a community over a critical inflection point), or might add up to significantly different patterns of risk-related behaviour is also of relevance and interest, although is probably virtually unanswerable. Very large scale behavioural studies, such as the Malawi national KAP, could potentially distinguish differences between ethnic groups, although these data were not reported from that study. Projects covering such a large geographic area, however, suffer from location as a significant confounder of ethnic effects.

There are a number of difficulties in comparing studies from different regions, not least the variations in ethnic structure. In Vietnam, most ethnic minority groups are poor and marginalised, with the majority Kinh comprising 85% or more of the population, and the largest minority about 1.4%. The populations of many sub-Saharan African nations are more evenly split between different ethnic groups, and numerically smaller groups may have higher social and economic status than the majority. Notwithstanding these caveats, those surveys which have compared majority and minority ethnic groups have consistently demonstrated lower risk behaviours in the majority (Ahmed 2001; Aikins et al. 1993; MacCormack et al. 1986), although this has not always fed through into lower prevalence (Ahmed 2001). Conversely, studies examining ethnic minority groups alone have usually

shown higher risk behaviour than that demonstrated in majority surveys (Sharma et al. 1993), although the latter have rarely been conducted in the same location, making direct comparison difficult.

Most of the research into the relationship between ethnic group and malaria has focused on potential genetic differences, and even the best reported studies mention behaviour only in passing to reassure the reader that there were no significant differences in malaria risk to account for the variation in prevalence (eg Modiano et al. 2001a). Numbers of subjects, methodologies and raw results are rarely reported, preventing the inclusion of these surveys in this analysis other than to highlight the requirement for KAP studies to be large and meticulous to have any chance of documenting inter-ethnic differences.

The absence of a readily identifiable hierarchy of risk amongst the minority ethnic groups in our study is thus not surprising, given published experience. The significant differences in malaria prevalence remain to be explained, however. Whilst the small sample sizes hinder the discovery of significant differences, unless the direction or relative magnitude of behavioural differences were to change markedly with increasing numbers, the lack of a consistent order of risk would remain. One possibility is the dominance of some behavioural effects over others. Published odds ratios for different risk factors are summarised in table 6.59. The purpose of this tabulation is to obtain a feeling for the order of magnitude of different risk factors, rather than conduct a meta-analysis. A number of the studies, particularly from Southeast Asia, are poorly reported, and, with a few notable exceptions, even the well reported studies tend not to give details of risk factors examined and found not to be statistically significant. The few studies comprehensively reporting their results often show no effect on malaria for variables demonstrated to be important in other studies (van der Hoek et al. 1998), especially after multivariate analysis (Guthmann et al. 2001; Koram et al. 1995), the results of which are virtually impossible to compare between studies. An exhaustive analysis of this body of work would require a separate

thesis, but a rapid inspection of the magnitude of odds ratios found for different risk factors suggests that only regular visits to the forest or bednets (in occasional studies) increase or decrease the risk of malaria by more than a factor of two. Very few individuals of any ethnic group in our study site visited the forest, and although the S'tiếng were significantly less likely to use bednets at home, this only accounted for 6-8% of individuals. Even if all these bednet non-users were parasitaemic all the time, this would not account for the interethnic differences in prevalence unless they were over-represented among survey subjects. Whilst the S'tiếng are the worst users of bednets in the home, the Nùng are less likely to use nets when sleeping in the field. Extrapolating from our data, the proportion of S'tiếng unprotected by bednets would be approximately 8% whilst sleeping at home, and 13% when sleeping in the field, whilst the equivalent values for the Nùng would be 0 and 20% respectively. On the basis of these figures alone, one would expect the Nùng to be at higher risk if a significant amount of time was spent sleeping in the field. This last variable was not measured systematically or accurately in this survey, neither was the seasonality of sleeping outdoors. It is likely that the reluctance of a minority of S'tiếng to use bed nets does have a significant impact on the prevalence of malaria in that community, but this doesn't appear to be sufficient to explain the differences recorded.

In the absence of major differences in dominant risk factors, is it still possible to account for the increased prevalence amongst the S'tiếng on behavioural grounds alone? None of the traits measured in this, or any, KAP study exist in isolation. Were large and detailed enough studies to be conducted to allow examination of combinations of behavioural traits or physical circumstances, it is possible that certain high or low risk patterns of behaviour would emerge. It is likely that these patterns would be specific to an ecosystem, however, which might be highly restrictive, or, at best, generalisable to a region. Any quest for such combinations should therefore start with an empirical examination of factors associated with malaria in the locality of interest, rather than attempting to extrapolate from published data. The prevalence in this survey was, however, far too low to reliably establish the local

importance of particular risk factors, and impossibly low if independence of ethnic group is to be documented. Table 6.58 is an attempt to force consensus between this survey, previous smear data on these survey subjects, and published results.

The loss of 10% of the smears in this survey to mislabelling was one of several operational issues. The question of whether a pilot study would have improved the quality of the study has been raised. Whilst this is almost invariably the case, such a pilot would have been extremely difficult in this setting. There were four significant methodological/operational issues which impinged on this survey's success: the failure to visit specified houses, the unexpected or uninformative response to some of the questions, the failure to complete the forms as specified, and the failure to label smears appropriately. Strenuous efforts were made to avoid the first of these, with confirmation of the existence of sample hamlets in advance, and, given that one of the major issues during the survey was actually getting to the houses, it is difficult to see that any pilot study would have ameliorated this issue. Piloting the study form would probably have resulted in certain questions being improved or dropped. The questionnaire had been designed in collaboration with researchers from NIMPE, however, and to insist on a pilot might have been seen as calling into question their judgement. The remaining issues are fieldworker dependent issues, and would have been very unlikely to have been affected by a pilot study, which might have been conducted with different groups of field workers, had it been possible, and the experience with previous surveys would suggest a minimal impact of exhortations to improve accuracy on field worker behaviour. Given the delays in implementing this study, a pilot was not, therefore, even considered.

Conclusion

This study failed to provide clear explanations of the interethnic variation in malaria prevalence. The resident Kinh did appear to have the lowest risk across all categories, but the effects were small. The only identifiable differences between the S'tiêng and other

ethnic groups were a greater degree of poverty and a slightly increased tendency to eschew bednets. Whilst the association between poverty and malaria may seem intuitive, studies attempting to demonstrate a relationship, particularly a quantitative relationship, have usually failed to do so. Bednets are clearly important, but whether the 7% difference in their use is sufficient to account for the observed variation in malaria prevalence is doubtful. It is possible that bednet use has recently increased amongst the S'tieng, and a KAP in 2000/1 would have yielded a different picture, but this is impossible to examine in retrospect. The only other behavioural trait consistently associated with an increased risk of malaria in the published literature is nocturnal forest activity. The results of this survey refute the assertions that the S'tieng, or other ethnic minorities in the study region, spend more time in the forest. Data collected at Dac O health station as part of another study suggest that the majority of forest workers in the study area are migrant Kinh, who would be missed by a population based survey (M. Chambers, personal communication).

The differences in malaria prevalence between ethnic groups remain to be convincingly explained, whilst at the same time there are sufficient differences in behaviour and socioeconomic status that the postulation of a putative unknown factor, such as an undetected additional genetic susceptibility trait, would be fanciful.

Trait	Association with malaria in this survey	Association with malaria in previous surveys	Association with malaria in published work	Probably significant?
Use of bednets at home	Protective at individual level (OR 0.26 (0.07-1.46)), not household level. Suggestion of trend to better protection with more recent treatment.	No protection (0/21 non-users smear positive vs 135/963 users, 0/10 non-using households vs 87/574 using). More recent treatment associated with increased prior risk of malaria.	Extensive evidence of superiority of treated nets over no nets. Good evidence of treated nets over untreated nets. Surprisingly conflicting evidence of untreated nets over no nets. No regional data on untreated nets vs no nets, but not using bednets a risk factor for clinical malaria in clinic or hospital based studies.	Yes
Use of bednets in the field	No protection	No protection	No systematic enquiry published, but suggestions that could be important as sleeping out away from home is important, but forest net use occasionally shown not to be protective	Uncertain
Number of people sharing bednet	No effect	No effect	No systematic enquiry published, but occasionally invoked as problem – esp with male female mixing taboos	No
Ratio of good to bad quality bednets	No effect	No effect	No systematic enquiry, but evidence that torn nets may act to keep mosquitoes in close proximity to victims and increase biting	No
Using free or purchased bednets	No effect	No effect	No enquiry published	Not in itself
Time at which household members usually retire	No effect	No effect	Not really amenable to intervention study, but has been quoted as possible reason for failure of nets (Leake et al. 1989)	No
Distance to working field	Significantly longer for smear positive individuals	No effect (mean non-significantly shorter for smear positive individuals)	Not reported	Uncertain

Table 6.58a. Summary of effects on malaria risk in this survey, in previous surveys and in the published literature, of factors in which the S'tiêng differ from other ethnic groups.

Trait	Association with malaria in this survey	Association with malaria in previous surveys	Association with malaria in published work	Probably significant?
House construction: Bamboo walls and/or thatched roof	Non-significant trend to susceptibility in thatched houses or those with bamboo walls, and to protection from wood construction.	Non-significant trend to protection by thatched roofs and bamboo walls, significantly increased risk associated with tiled roofs disappeared on adjustment for ethnic group	Inconsistently associated with transmission, but very different criteria used in different studies.	No
Livestock ownership: cattle Pigs Any	All significantly protective	Cattle ownership significant risk in unadjusted analysis, no effect when adjusted for ethnicity. Similarly for any animal. Pig ownership no effect.	Zooprophylaxis contentious. Could increase or decrease risk in this environment. See text.	No
Farming techniques: Cashew Pepper Rice	Pepper protective, just fails to reach significance. Effect holds across ethnic groups.	Pepper & coffee protective, rice and cashew farming increase risk. Adjustment for ethnic group abolishes coffee effect, and decreases effects of other cropping patterns – cashew becomes NS	Most published work has focused on irrigation. Usually found that areas with paddy can be less prevalent. Little or no work on individual farming methods. See text. (Ijumba et al. 2001; Marrama et al. 2004)	No
Health care seeking behaviour	A significantly lower risk for individuals having to pay for treatment during their last malarial illness No difference in risk of different durations of treatment of previous malarial illness. Conflicting results between household and individual datasets wrt risk associated with health care choices	No effect of having had to pay for treatment during their last malarial illness (and OR's >1) Similarly no difference No significant effects of provider choice. OR's generally more consistent in direction.	More often associated with malaria outcome in published studies	No

Table 6.58b. Summary of documented effects of factors in which the S'tiêng differ from other ethnic groups (continued).

Trait	Association with malaria in this survey	Association with malaria in previous surveys	Association with malaria in published work	Probably significant?
Economic status: Annual income Number of months without sufficient rice	No association between income and malaria, or between ownership of certain consumer durables and malaria. No effect of number of months without sufficient rice.	No significant association between income and malaria. Borderline significant increased risk from owning Honda becomes significant on adjusting for ethnic group. No effect of number of months without sufficient rice.	Relationship between individual or household economic status and malaria inconsistent. More reliable association between economic status of region/area and prevalence	No
Education	No significant effect of any indicator of education on malaria	No significant effect of any indicator of education on malaria	Inconsistent association between individual or maternal education and prevalence	No
Knowledge of malaria transmission and treatment	No significant effect of professing to know how malaria is transmitted, believing malaria is preventable, or knowing antimalarials are free	No effect	Again, not as consistently related to prevalence as might be expected	No

Table 6.58c. Summary of effects on malaria risk in this survey, in previous surveys and in the published literature, of factors in which the S'tiếng differ from other ethnic groups.

Cat-egory	Population / endemnicity	Criterion	OR (95% CI) or specified index	Reference
Bednet ownership, use and care	Mostly Sub-Saharan Africa	Overall PE of ITN's against parasitaemia compared with no nets	EIR>1: 13% EIR<1: 42%	(Lengeler 2004)[1]
	Refugee population in NE Thailand. Treated vs untreated.	Summary of PE against incident and point prevalent parasitaemia	38% falciparum No effect on vivax	(Luxemburger et al. 1994)[2]
	Local in-migrants to district in SE Thailand. Treated vs untreated.	Individuals smear positive Episodes of smear positivity	0.75 (0.40-1.39) NS 0.59 (0.36-0.95)	(Kamol-Ratanakul et al. 1992)[3]
	Thailand Hospital based case control study.	Irregular net use No use of nets	1.71 (1.05-2.79) 2.68 (1.03-6.99)	(Fungladda et al. 1987)[4]
	Thailand Clinic based study	Irregular net use	1.52 (1.01-2.29)	(Fungladda et al. 1986)[5]
	Sri Lanka Population based cohort study.	Clinical malaria episodes in individuals with bed nets (25) vs those without (255)	0.16 (0.05,0.45)	(van der Hoek et al. 1998)[6]

[1]) The Cochrane review of ITN intervention studies. Almost all studies included in this review were from Sub-Saharan Africa. Summary of results by EIR, as efficacy varied with endemnicity. The PE for clinical malaria episodes was higher than that against parasitaemia alone in regions with EIR greater than 1 infected bite/person/year.

2) RCT of ITN's in Karen school children. Randomised individually, so no geographic separation between groups. Compliance monitored. Outcomes were incidence of parasitaemic episodes during passive case finding and prevalence in 2 surveys. Well reported.

[3]) RCT of ITN's (126) vs untreated nets (135) in Bothong district 85km south east of Bangkok. Household level randomisation. Subjects were all in migrants, resident in study area for at least 6 months, and were followed for 35 weeks with weekly blood smears and net compliance checks. Methods well reported, only summary results reported.

[4]) Hospital based case control study. All 210 smear positive and 210 unmatched smear negative hospital patients reporting no episodes of malaria in previous 12 months. 80% inpatients in both groups. Results not reported in easily interpretable manner, not clear whether only adults were included, though average age would make that seem likely. OR are those presented from multivariate analysis, which included age, sex, education and district and duration of residence in each model, plus the variable given. Living and working in the forest refers to the period 2 weeks prior to admission. Poorly reported.

[5]) 200 smear positive cases presenting to health station and 200 smear negative unmatched controls who reported not having had malaria in previous year. Baseline API 50-70/1000 pop/year. Income borderline significant, malaria knowledge no effect. Conducted during rainy season. Incompletely reported.

[6]) Cohort of 280 residents in one Sri Lankan village with unstable, seasonal transmission. Village mapped and data on environmental and risk factors gathered. Cohort followed every 2 days for a year with self reporting of malaria episodes diagnosed at a local health care facility. Well reported.

Cat-egory	Population / endemnicity	Criterion	OR (95% CI) or specified index	Reference
Bednet ownership, use and care	Malaysia ITN's vs no nets.	Parasitaemia prevalence	Initial fall followed by rise to baseline after 6 months	(Hii et al. 1987)[7]
	Central China Treated vs untreated.	Protective efficacy all ages (incidence) Protective efficacy in children (prevalence)	43% 75%	(Luo et al. 1994)[8]
	Solomon Islands. ITN's vs DDT vs no intervention	Parasite prevalence in children (cross section)	No significant differences – see footnote	(Hii et al. 1993)[9]
	Irin Jaya, Indonesia (hyperendemic) Treated vs untreated.	Protection during 2nd year of study: Children (<10yrs) Adults (>10yrs)	 0.39 (0.25-0.60) 0.37 (0.24-0.54)	(Sutanto et al. 1999a; Sutanto et al. 1999b)[10]
	East Flores, Indonesia Figures given are for ITN vs no nets	SPP in surveys after: 5 months 10 months 16 months	 0.56 (0.26-1.19) 0.49 (0.29-0.83) 0.33 (0.16-0.65)	(Nalim et al. 1997)[11]

7) Small scale cluster trial in Malaysia (306 nets in 139 households in 5 villages vs control village (?numbers)) of one off mass drug administration vs one off drugs and ITN's
8) Intervention trial of treatment of villager's own nets. 11200 in 4 townships vs 2269 in one. Low prevalence, mostly *P.vivax*. Active and passive case finding of clinical malaria cases over 6 months of a transmission season, and 3 surveys of selected cohort of 424 and 155 intervention and control children under 10. Reasonably well reported.
9) ITN (580 people in 16 villages) vs residual spraying (644 in 30) vs control (438 in 17) followed for 18 months with surveys of children from 1-9 years old (only small numbers). Trend to spraying>control>nets (46% of 53, 29% of 34, 21% of 29).
10) ITN (237 children and 420 adults in 158 households in one village) vs untreated nets (201 children and 364 adults in 141 households in the other) in 2 villages 2km apart. 277 impregnated and 261 untreated nets distributed, which were changed every four months. Four surveys per year, each covering only 50% of study population in each village. Unclear whether self or randomly selected subsample. Effect slow to appear, and differences between villages appeared after big rains, although differences seemed greatest at end of small rains, not big rains. ITN's also reduced level of parasitaemia in the second year. Benefits in children lagged behind those in adults, unusually. Reasonably reported apart from survey methods.
11) Trial of ITN's vs residual spraying vs no intervention in one village, pop approx 1000, 2 villages, pop approx 2000 and one village, pop approx 330, respectively. Populations are approximate as considerable variation over course of the study. 578 nets distributed, reimpregnated every 4-6 months. Malariometric surveys every 6 months: no mention made of sampling strategy, but only 100-200 individuals/area included in each survey. Effect of spraying similar in children and adults, effects of bednets greater in children. Effect on vivax greater than that on falciparum. Reasonably reported except for survey sampling strategy.

Cat-egory	Population / endemnicity	Criterion	OR (95% CI) or specified index	Reference
Bednet ownership, use and care	Burma See footnote	Incidence of clinical malaria at local clinic Prevalence in bimonthly surveys	Average PE 50% Average PE 65%	(Lwin et al. 1997)[12]
	Assam, India	Average monthly parasite index after 18 months of intervention Treated vs no nets Untreated vs no nets Treated vs untreated nets	 0.14 (0.09-0.20) 0.62 (0.42-0.79) 0.22 (0.15-0.32)	(Jana-Kara et al. 1995)[13]
Field and forest activity	Northern Thailand	SPP in visitors to forest vs SPP in all inhabitants (see note)	27.6	(Butraporn et al. 1995)[14]
	Thailand Hospital based case control study.	Living in forest: Univariate Corrected Working in forest: Univariate Corrected	 5.67 (3.49-9.40) 10.25 (6.28-16.7) 2.36 (1.47-3.82) 7.19 (4.41-11.73)	(Fungladda et al. 1987)[4]
	Thailand	Live in forest Slept outside recently	6.29 (1.56,25.42) 4.13 (1.29,13.13)	(Chaveepojnkamjorn et al. 2004)[15]
	Thailand	Forest goers	8.5 (5.52-13.5)	(Butraporn et al. 1986)[16]
	Thailand	Live in forest Work in forest[17]	2.37 (1.51-3.71) 1.3(0.73,2.45)NS	(Fungladda et al. 1986)[5]

[12]) Chemoprophylaxis with SP (440) vs SP and ITN and treated scarves and wrist bands (540) in 2 areas 3 miles apart. Migrants excluded. Treatment of family's own nets (proportion of control group with own net unclear). Followed for 6 months through one peak transmission season with bimonthly surveys and monitoring of cases presenting to local health care centre. Not very good study not very well reported.

[13]) ITN's in 3 villages (pop 1800), untreated nets in 6 villages (pop 1500) and 3 control villages (pop 1800), clusters 3-9km apart, pop mostly ethnic minorities. Weekly active surveillance visiting houses and taking smears from any febrile inhabitants. DDT residual spraying withdrawn a year prior to collection of baseline data and 2 years prior to intervention. Nets reimpregnated after 8 months. Well reported.

14) Poor cross section/mini-cohort design in 4 Thai villages Any smear positive from smears taken at any time from forest visiting population (including study smears on return from each forest visit) vs overall prevalence in villages in one off survey (seasonality not specified) (60% of 1083 slides from 729 forest workers positive cf 5.3% of all inhabitants positive in single survey)

15) Case control study of foreign/migrant workers attending Thai government health clinics. 217 malaria cases vs 217 clinic attenders without malaria.

16) 349 cases retrospectively selected from 1511 cases presenting to health services and 349 prospectively recruited, age, sex and village of residence matched controls (sampling frame and methods unreported). Crude, unmatched odds ratios calculated from data presented, matched data not given. Poorly reported.

[17]) Living or working in forest in 2 weeks prior to clinic attendance.

Cat-egory	Population / endemnicity	Criterion	OR (95% CI) or specified index	Reference
House construction and location	Sri Lanka.	Brick and tile vs mud and thatch.	PE approx 50%	(Gamage-Mendis et al. 1991)[18]
	Peru Population based case control study	Distance to nearest canal (cf <100m) 100-199m >=200m	 0.67 (0.45-1.01) 0.39 (0.27-0.57)	(Guthmann et al. 2001)[19]
	Peru	Brick vs other materials (univariate)	0.55 (0.36, 0.83)	(Guthmann et al. 2001)[19]
	The Gambia Severe malaria case control study	>4 people sleeping in same room	1.8 (1.1-2.96)	(Koram et al. 1995)[20]
	Bacan island, Indonesia Small case control study	"Temporary" vs "permanent" housing >4 members in household (regardless of house size)	8.72 (1.2-386.2) 2.53 (0.98-6.64)	(Roosihermiatie et al. 2000)[21]
	Orissa, India	Thatched roof vs tiled roof and brick house: with false ceiling without false ceiling	 6.7 11.3	(Subramanian et al. 1991)[22]

18) Cohort study over 17 months in one village in general house construction – Malaria incidence rate (?clinical?routine smear) 21.2% in mud houses vs 10.5% in brick (?numbers of each?)

19) Population based case control study in low transmission area of Peru. Active case finding and age- sex- and village matching to 3 community controls from census list. 323 case and 1169 controls. OR's reported are those estimated by multiple conditional logistic regression. Well and fully reported.

[20]) Hospital recruited severe malaria cases (192) or health station recruited mild malaria cases (192) (all children) compared with 384 age and location matched control living >400m from case in peri-urban areas. All results reported in this table are OR's estimated through multiple conditional logistic regression. House construction only significant in univariate analysis. Well reported.

[21]) 571 self selected individuals from pop of 17746 in 11 villages in Bacan island, Indonesia. Participants had suffered fever in last week (211) or not experienced fever in last month (360). 93 febrile and 45 afebrile subjects were parasitaemic (mostly *P.vivax*). 3 cases excluded leaving 90. 90 controls selected. Cases and controls each supplemented with 10 subjects from local HC. "Temporary" housing was usually wood and thatch, "permanent" housing brick and tin. HC use, net use, income, education all no difference. Not very good study, fully reported.

[22]) Cohort of 1476 individuals in 316 houses in 1 village in Orissa. 296/740 smears positive over course of a year from 253 individuals. Binomial model of area of village, cattle to man ratio, cattleshed proximity and house construction: OR's given have been estimated from this model. Negative association between malaria incidence and cattle to human ratio in sections of village found, but details of this effect not reported. Seems to be a reasonable study, but results poorly reported.

Cat-egory	Population / endemnicity	Criterion	OR (95% CI) or specified index	Reference
House construction and location	Sri Lanka	Mud & thatch vs brick & tile/ corrugated iron.	1.74 (1.14-2.65)	(Van Der Hoek et al. 2003)[23]
	Sri Lanka	<750m from stream vs >=750m	5.93 (3.50-8.91)	(Van Der Hoek et al. 2003)[23]
	Vietnam	Thatched roof.	1.39 (1.00-1.94)	(Khai et al. 2000)[24]
	Sudan	>2 rooms in house >5 people in house	0.6 (0.37-0.98) 2.5 (1.4-4.5)	(el Samani et al. 1987)[25]
	Northern Thailand	House close[26] to: Forest Stream Forest and stream	 20(13.3-32.3) 6.3 (3.4-11.7) 28.5 (10.3-38.9)	(Butraporn et al. 1986)[16]
Occupation and farming methods	Peru	Individual engaged in agricultural work	0.52 (0.31-0.86)	(Guthmann et al. 2001)[19]
	Ethiopia	No livestock vs separate pen	15% vs 23.8%	(Seyoum et al. 2002)[27]
	Orissa, India	Cattle under same roof, in compound or outside compound Cattle to human ratio	No difference See note	(Subramanian et al. 1991)[22]
	The Gambia Paired "cohort"	SPP in compounds with or without cattle. Unadjusted Adjusted for wealth	 Lower SPP with cattle (NS) Higher SPP with cattle (NS)	(Bogh et al. 2002)[28]
	Northwest Pakistan	SPP in households owning cattle vs those not owning cattle	1.66 (1.22-2.25)	(Bouma et al. 1995)[29]

[23]) 2 year nested case control study in superset of study 5. 219 malaria cases presenting to one of the local health stations. 4 community controls selected for each case from census list (656 total). OR's reported are adjusted for age, use of coils, smoke or bednets, DDT spraying, house construction or distance to stream as appropriate, distance to cattleshed (non-significant in itself once adjusted) and sex. Adequately reported.

[24]) Cross sectional cluster survey of 2441 individuals in two border provinces of north central Vietnam. Smear taken and information on malaria symptoms in previous year collected. Very low SPP. Unclear if data on risk factors gathered on all subjects or just subset of 729 adults. OR's refer to REPORTED SYMPTOMS OF MALARIA only. CI estimated assuming all 2441 individuals gave data on housing. Poorly reported.

25) Cross sectional survey of 445 under 5's in Sudan. Malaria diagnosis based on retrospective history only. Detailed information on inhabitants/room not available.

26) Distance to forest or stream not specified

27) Cross section comparison of SPP in children in houses with livestock in house, separate pen, and no livestock (livestock in house was highest).

28) Children near cattle randomly selected and paired with age and bednet use matched controls sleeping >50m from cattle in same village. Total of 29 villages and 102 pairs. Significant difference in number with high parasitaemia also disappeared on correcting for wealth. Well reported.

Cat-egory	Population / endemnicity	Criterion	OR (95% CI) or specified index	Reference
Malaria knowledge and HC seeking behaviour	Thailand	Knowledge of malaria transmission: Univariate Corrected with either: bednet use *or* forest contact in the model	 0.58 (0.36-0.91) 0.54 (0.37-0.79) 0.55 (0.37-0.82)	(Fungladda et al. 1987)[4]
	The Gambia	Maternal knowledge of malaria	0.68 (0.48-0.95)	(Koram et al. 1995)[20]
	Southern Mexico	No use of formal health services Poor knowledge of malaria transmission and treatment	4.69 (3.01-7.29) 2.3 (1.3-4.07)	(Danis-Lozano et al. 1999)[30]
	Mali Severe malaria case control study	Maternal education Maternal knowledge of malaria transmission	0.52 (0.31-0.86) 0.46 (0.25-0.86)	(Safeukui-Noubissi et al. 2004)[31]
Socioeconomic status	Peru	Primary education Secondary education	0.71 (0.50-0.99) 0.40 (0.29-0.71)	(Guthmann et al. 2001)[19]
	The Gambia	Own refrigerator[32] Crowding – see above	0.43 (0.27-0.69)	(Koram et al. 1995)[20]
	Indonesia	Large family – see above		(Roosihermiatie et al. 2000)[21]
	Sudan	Own fridge Crowding - see above	0.5 (0.36-0.94)	(el Samani et al. 1987)[25]
	Northern Thailand	Lowest income group[33] vs remainder Highest group vs rest	2.3 (1.7-3.2) 0.35 (0.24-0.52)	(Butraporn et al. 1986)[16]
	Northern Thailand	Education: Primary vs none Any vs none Secondary vs rest	 No difference No difference 0.4 (0.20-0.75)	(Butraporn et al. 1986)[16]

[29]) Cross sectional survey of 2042 schoolchildren in 2 areas. Children were asked whether their family owned cattle, and bled for thick smear. Responses were not validated, and distance between house and cattle not documented. Stratified OR by village and nationality (Afghan or Pakistani). Adequately reported.

[30]) Cross sectional survey of 7628 individuals. Malaria diagnosed on the basis of positive *P.vivax* serology plus history of symptoms plus record of positive blood smear at local health services, compared with individuals with negative serology. Adequately reported.

[31]) Matched case control study of 130 severe malaria cases (all children) and 260 community controls without a history of severe malaria matched for age, residence and duration of residence. Interviews and control collection during follow up visits. OR reported are from a stepwise derived conditional logistic regression model. Well reported.

[32]) Ownership of a radio, motorbike or bicycle were not associated with malaria. Fewer households owned a refrigerator than any of these items.

[33]) Annual monthly income categorised into bands of US$ <1154, 1154-2307, >2307.

Cat-egory	Population / endemnicity	Criterion	OR (95% CI) or specified index	Reference
Migration	Southern Mexico	Not born in village of current residence	11.67 (5.21-26.11)	(Danis-Lozano et al. 1999)[30]
	Northern Thailand	Duration of residence <=5yrs vs >5yrs >10yrs vs <=10yrs	1.53 (1.12-3.09) 0.60 (0.42-0.84)	(Butraporn et al. 1986)[16]
	Thailand	Duration of residence (months) in study area in univariate analysis	Cases less than controls (but details not given)	(Fungladda et al. 1987)[4]
	Thailand	Born outside province under study	Cases less than controls (but details not given)	(Fungladda et al. 1986)[5]

Table 6.59. Selection of published data on major categories of social and environmental malaria risk factors tested in this survey.

Chapter 7 – Case Control Study

Introduction

Severe malaria remains our best surrogate of malaria mortality in the era of effective treatment. Case control studies of cerebral malaria, severe anaemia and other manifestations of severe malarial disease have provided the most compelling clinical data on the selective advantage of the haemoglobinopathies.

There are a number of methodological problems inherent in the case control study design, which have been discussed in chapter 2. Details of the protocol for this study are also presented therein, but in brief: patients of all ages meeting a fairly relaxed definition of severe malaria were recruited as cases at 4 centres. Controls were prospectively recruited from the environs of the case's dwelling, and were additionally matched for age, sex and ethnic group. Two controls were recruited for each case. Parents of the cases were sought as genetic controls, and if both parents were not available, as many siblings as possible were recruited.

This study, as others, involved the efforts of many. The patients were recruited by doctors at the participating centres. Dr Cao Quang Thái acting as a principal point of liaison for Đồng Xoài, and latterly for Phước Long as well, particularly with regard to recruiting community controls. He was also responsible for recruiting many of the community controls for patients recruited at HTD. The community controls in Phước Long and Đồng Phú were recruited by the district and provincial malaria control teams, respectively. All slides were re-read by Miss Ly and her team at HTD. Haemoglobin genotyping by HPLC was carried out initially in John Clegg's lab by Katie Miles and subsequently Angela Allen, and latterly Chris Fisher. The α thalassaemia genotyping has been conducted in Vietnam by Nguyễn Thị Ngọc Quyền, with initial assistance from Vũ Thị Hằng, under the

supervision of Sarah Dunstan. Data was entered by Miss Tâm. My role was to design the study, design the study forms, supervise the study conduct, and analyse the data.

Results

Case recruitment

A total of 343 cases were recruited between all four centres between June 2000 and August 2004. The contributions of the various centres are shown in table 7.1, with a graphical depiction of the time course of recruitment depicted in fig 7.1 (the one case in Bù Đăng was recruited in November 2004).

A total of 25 cases were excluded. Three cases were excluded due to the absence of controls: in one instance this was due to inadequate address details, in a second to the control gathering team being unable to confirm that the case had lived in that vicinity, and in a third to members of the local community all refusing to participate unless they were paid. Twenty two cases were excluded for not actually meeting admission criteria. All but one were individuals entered on the basis of hyperparasitaemia whose parasite counts were subsequently demonstrated not to reach the threshold. This problem was particularly evident in Phước Long, where, despite considerable persuasion, slides were not counted properly, and individuals scored as 4+ were all recruited, despite the cut off for this score being approximately 30-50,000 parasites/μl. The remaining individual was admitted in coma, and a blood smear read as positive in Phước Long was negative at HTD (as were HRP2 testing and plasmodium PCR).

Centre	Total number of cases recruited	Number of Kinh cases recruited	Number of S'tiêng cases recruited
Phước Long	123	89	30
HTD	78	76	0
Đồng Xoài	141	93	25

Table 7.1. Breakdown of case recruitment by centre

Demographics

The average age of cases was 22.8 years old. This aggregate figure disguises considerable variation between centres, with almost all HTD admissions being adult (see note on referral patterns in Chapter 2): the average age of HTD admissions was 33 years, compared to 19.1 and 19.5 for Đồng Xoài and Phước Long respectively (table 7.2). The majority of cases were Kinh (75%), followed by the S'tiếng (17%). Once again, there was a significant difference between centres: all but two of the cases recruited at HTD were Kinh (the exceptions both being Tày), whilst 25% of patients in Phước Long, and 20% in Đồng Xoài, were S'tiếng (table 7.3). There were striking differences in the age distribution of cases from different ethnic groups: 81% of S'tiếng cases were under 10, compared to 21% of the Kinh cases recruited at Phước Long and Đồng Xoài (table 7.4). Missing data accounts for the slight differences in the denominators in these tables.

Age group	Phước Long	HTD	Đồng Xoài	Total
<2	4 (4%)	0 (0%)	5 (4%)	9 (3%)
2-4	15 (14%)	2 (3%)	19 (15%)	36 (11%)
5-9	24 (22%)	0 (0%)	18 (14%)	42 (13%)
10-14	5 (5%)	1 (1%)	13 (10%)	19 (6%)
15-19	12 (11%)	7 (9%)	15 (12%)	34 (11%)
20-29	19 (17%)	29 (37%)	28 (22%)	76 (24%)
30-39	16 (15%)	12 (15%)	9 (7%)	37 (12%)
40-49	7 (6%)	15 (19%)	9 (7%)	31 (10%)
50-59	5 (5%)	8 (10%)	5 (4%)	18 (6%)
60-69	1 (1%)	3 (4%)	1 (1%)	5 (2%)
70+	2 (2%)	2 (3%)	5 (4%)	9 (3%)
Total	110 (35%)	79 (25%)	127 (40%)	316

Table 7.2. Age distribution of sample with breakdown by centre

Ethnic group	Phước Long	HTD	Đồng Xoài	Total
Kinh	80 (73%)	75 (97%)	80 (63%)	235 (75%)
S'tiếng	26 (24%)	0	26 (20%)	52 (17%)
Tày	2 (2%)	2 (3%)	5 (4%)	9 (3%)
Nùng	1 (1%)	0	2 (2%)	3 (1%)
Khmer	0	0	10 (8%)	10 (3%)
Dao	0	0	3 (2%)	3 (1%)
Hoa	0	0	1 (1%)	1 (0%)
Total	109 (35%)	77 (25%)	127 (41%)	313

Table 7.3. Ethnic group of cases, by centre

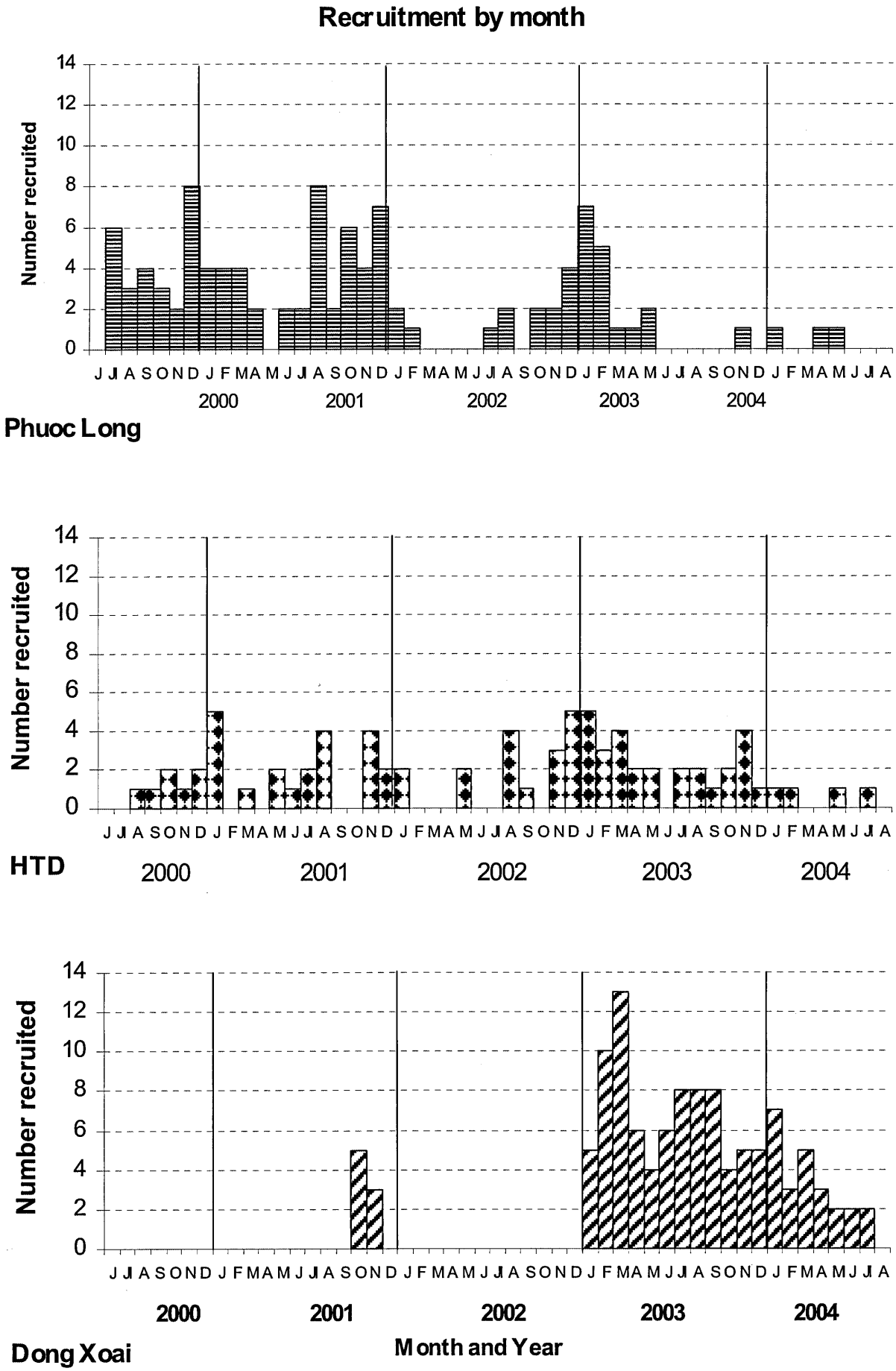


Fig 7.1. Recruitment by centre by month.

Age group	Kinh	S'tiếng	Tày	Nùng	Khmer	Dao	Hoa	Total
<2	3 (1%)	5 (10%)	0	0	1 (10%)	0	0	9 (3%)
2-4	11 (5%)	19 (37%)	1 (11%)	0	3 (30%)	1 (33%)	0	35 (11%)
5-9	21 (9%)	18 (35%)	0	0	2 (20%)	1 (33%)	0	42 (13%)
10-14	15 (6%)	3 (6%)	0	0	1 (10%)	0	0	19 (6%)
15-19	30 (13%)	1 (2%)	1 (11%)	0	2 (20%)	0	0	34 (11%)
20-29	63 (27%)	5 (10%)	4 (44%)	2 (67%)	1 (10%)	0	0	75 (24%)
30-39	35 (15%)	0	0	1 (33%)	0	0	1 (100%)	37 (12%)
40-49	28 (12%)	1 (2%)	2 (22%)	0	0	0	0	31 (10%)
50-59	18 (8%)	0	0	0	0	0	0	18 (6%)
60-69	5 (2%)	0	0	0	0	0	0	5 (2%)
70+	6 (3%)	0	1 (11%)	0	0	1 (33%)	0	8 (3%)
Total	235 (75%)	52 (17%)	9 (3%)	3 (1%)	10 (3%)	3 (1%)	1 (0%)	313

Table 7.4. Age group distribution of cases by ethnic group

Cases were predominantly male (58.3%, p=0.003). This effect, although seen across all age groups, was more pronounced amongst adults (table 7.5), and Kinh (table 7.6). An extraordinary 73% of cases were in-migrants to their current district of residence. The S'tiếng are all indigenous, leaving 86% of Kinh cases as migrants. This compares with only 52% of Kinh survey subjects, suggesting that being a migrant might be a risk factor for severe disease. 81% of Kinh community controls were migrants, however, so this effect is not established. The method of selection of controls might have overmatched with regard to answering this question. Kinh cases had spent a considerably shorter time in their district of residence than controls (5.35 years vs 12.9 years, p<0.0001) , however. This did not hold for the other ethnic groups (7.4 years vs 7.6 years, p=0.85).

Age group	Sex	
	Male	Female
<2	67% (8)	33% (4)
2-9	52% (41)	48% (38)
10-19	53% (27)	47% (24)
20-39	62% (73)	38% (45)
40-59	70% (35)	30% (15)
60+	44% (4)	56% (5)
Total	59% (188)	41% (131)

Table 7.5. Sex distribution by age group. Percentage (number). There is a greater preponderance of men amongst adult cases (under 15's vs over 15's p=0.04)

Age group	Ethnic group			Total
	Kinh	S'tiếng	Other	
<2	100% (3)	50% (6)	67% (3)	67% (12)
2-9	42% (33)	53% (36)	78% (9)	51% (78)
10-19	63% (38)	0 (4)	38% (8)	54% (50)
20-39	65% (97)	50% (2)	50% (18)	62% (117)
40-59	73% (44)	100% (1)	25% (4)	69% (49)
60+	44% (9)	0	0	44% (9)
Total	63% (224)	49% (49)	52% (42)	59% (315)

Table 7.6. Proportion of male subjects (total subject number) by age and ethnic group. The Kinh appeared to have a greater preponderance of men but this effect was not significant (p=0.11).

Prior treatment

Nearly 72% of cases recruited at Đồng Xoài and Phước Long had received some sort of treatment in the 2 weeks prior to admission (data was not gathered at HTD). There were no differences between centres, but Kinh subjects were slightly more likely to have sought prehospital treatment (table 7.7). Younger children appeared less likely to have received healthcare than older children and adults (table 7.7), the effect nearing significance in the ethnic minority groups. Kinh parents were more likely than those from ethnic minorities to have sought care for their children (82% vs 54% of subjects under 10, p=0.007).

Age group	Ethnic group			Total
	Kinh	S'tiêng	Other	
<2	100% (3)	50% (6)	50% (2)	64% (11)
2-9	80% (30)	58% (36)	40% (10)	64% (76)
10-19	71% (31)	75% (4)	75% (8)	72% (43)
20-39	79% (58)	100% (2)	69% (13)	78% (73)
40-59	81% (21)	100% (1)	67% (3)	80% (25)
60+	75% (4)	(0)	(0)	75% (4)
Total	78% (147)	61% (49)	61% (36)	72% (232)

Table 7.7. Percentage of individuals receiving treatment in the 2 weeks prior to admission, by age and ethnic group. Total number in age/ethnic category in parentheses. Kinh vs S'tiêng p=0.004. Under vs over 10's in non-Kinh p=0.007.

Details of healthcare providers used were also sought, but this section was particularly poorly completed. The available data are presented in table 7.8. There was insufficient data to analyse relationships between age or ethnicity and choice of healthcare provider.

There was no association between severity of disease and previous treatment, even when the definition of previous treatment was limited to healthcare outlets which might be expected to provide efficacious treatment (tables 18 and 19, appendix 2). The healthcare provider/treatment modality section was presented as a series of questions allowing yes/no responses (and thus multiple positive answers). In addition to the generally poor completion rate for these questions, we had to assume that a null response to any question in this set was equivalent to a negative response if there were any positive responses in the

set in any given case record, in order to combine data from the different questions. This assumption may not be valid.

Treatment provider or type	Percentage using treatment provider or type	Number of completed forms (max 239)
Hospital	37%	70
Health station	55%	87
Market trader	75%	83
Private doctor or pharmacist	65%	91
Traditional herbal treatment	8%	60
Traditional physical treatment (eg cupping)	27%	60

Table 7.8. Treatment provider usage

Clinical features

The frequencies of entry criteria clinical syndromes are given in table 7.9. Many individuals met several different severity criteria, especially at HTD. The cases meeting only one entry criterion were predominantly either hyperparasitaemic or suffering from cerebral malaria (table 7.10). The relatively large number of cases with severe anaemia and no other criteria recruited at Đồng Xoài is likely to reflect referral patterns rather than any true variation in disease presentation, as Đồng Xoài is the only hospital in Bình Phước with the capacity to transfuse blood. The larger number of cases with more than one syndrome recruited at HTD probably also reflects referral patterns: the only absolute indication for referral is renal failure, as very few, if any, provincial hospitals have haemofiltration capability. Deeply comatose patients and those with shock refractory to initial resuscitation or multiorgan involvement are also more likely to be referred. The lower proportion of cases with only one qualifying syndrome recruited at HTD might be expected if one considers that renal failure is often accompanied by acidosis, shock by

hyperlactataemia and acidosis, and multiorgan involvement by definition meets more than one criterion. Hyperparasitaemia or jaundice alone are very unlikely to be referred.

Clinical syndrome	Phước Long (110)	HTD (79)	Đồng Xoài (127)	Total (316)
Anaemia	14 (13%)	6 (8%)	27 (21%)	47 (15%)
Jaundice	36 (33%)	49 (62%)	14 (11%)	99 (31%)
Cerebral	54 (49%)	44 (56%)	45 (35%)	143 (45%)
BWF	14 (13%)	16 (20%)	15 (12%)	45 (14%)
Bleeding	2 (2%)	5 (6%)	4 (3%)	11 (4%)
Renal failure	0	18 (23%)	6 (5%)	24 (8%)
Respiratory distress	2 (2%)	19 (24%)	9 (7%)	30 (10%)
Hyperparasitaemia	41 (37%)	18 (23%)	49 (39%)	108 (34%)
Shock	1 (1%)	2 (3%)	0	3 (1%)
Acidosis	0	4 (5%)	0	4 (1%)
Hyperlactataemia	9 (8%)	14 (18%)	4 (3%)	27 (9%)

Table 7.9: Frequency of severity syndromes at the different centres. Column headings contain recruitment centre (number of cases), cell contents number (percentage) of cases meeting that criterion.

Clinical syndrome	Phước Long (110)	HTD (79)	Đồng Xoài (127)	Total (316)
Anaemia	2 (2%)	1 (1%)	18 (14%)	21 (7%)
Jaundice	8 (7%)	8 (10%)	5 (4%)	21 (7%)
Cerebral	27 (25%)	11 (14%)	28 (22%)	66 (21%)
BWF	4 (4%)	5 (6%)	0	9 (3%)
Bleeding	1 (1%)	0	1 (1%)	2 (1%)
Renal failure	0	0	0	0
Respiratory distress	0	0	2 (2%)	2 (1%)
Hyperparasitaemia	13 (12%)	3 (4%)	32 (25%)	48 (15%)
Shock	0	0	0	0
Acidosis	0	0	0	0
Hyperlactataemia	0	0	1 (1%)	1 (<1%)
Total	55 (50%)	28 (35%)	87 (69%)	170 (54%)

Table 7.10: Frequency of severity syndromes in cases with only 1 qualifying feature. Cell contents as table 7.9.

Age and presentation

Clinical syndromes have been associated with different age groups. These associations have not always been consistent, and have varied with transmission intensity. Our expectations were that cases with renal failure would be older, as we rarely see this complication in children, and hyperparasitaemia would be more common in children.

Crude analysis of the data in this study would appear to show that jaundice, cerebral malaria, renal failure, acidosis, respiratory distress and hyperlactataemia are more common

with increasing age, whilst the opposite trend holds for hyperparasitaemia and anaemia (Table 20 appendix 2). These interpretations are confounded by centre, however, as very few children were recruited at HTD (3 cases under 16 years of age). Examining centres individually removes most associations (table 7.11): cases with jaundice at HTD were significantly older than those without (36.2 vs 28.4 p=0.019), but those at Đồng Xoài were non-significantly younger (18.8 vs 26.4 p=0.10); the anaemia effect is dominated by Đồng Xoài, where anaemic cases were younger than others (13.3 vs 21.3 p=0.02), whilst no differences were apparent at HTD or Phước Long. A similar difference in direction of age trend is seen for haemoglobinuria (26.0 vs 35.2 p=0.02 at HTD, 21.2 vs 18.9 at Phước Long, 21.3 vs 19.3 at Đồng Xoài). Congruent but non-significant trends were seen for hyperparasitaemia (negatively associated with age) and hyperlactataemia (positively associated with age), the latter reaching significance at HTD. Analysing Phước Long and Đồng Xoài together revealed a significant positive association with age for renal failure and respiratory distress (table 21 appendix 2): only 6 cases suffered renal failure, but only one of these was a child. These trends were not seen at HTD (table 22 appendix 2). Examining children and adults separately (tables 7.12 and 7.13), regardless of centre, showed a positive association with age for hyperlactataemia and respiratory distress amongst adults, and a positive association with age for jaundice in children. More children (<16) at Phước Long and Đồng Xoài had hyperparasitaemia than adults, and a significantly lower proportion of younger children (<10) were jaundiced (table 23 and 24, appendix 2). Although numbers are small, and confounding by centre is large, it seems reasonable to state that in this case series the risk of hyperparasitaemia decreases with age, the risk of jaundice or hyperlactataemia increases with age, and there is no relationship between age and cerebral malaria.

Syndrome	Centre	Number of cases with syndrome	Mean (SEM) age of cases with syndrome	Number of cases without syndrome	Mean (SEM) age of cases without syndrome	p value
Severe anaemia	Phước Long	14	19.5 (4.3)	94	19.1 (1.6)	0.94
	HTD	6	33.3 (5.5)	72	33.3 (1.7)	1.00
	Đồng Xoài	27	13.3 (2.2)	97	21.3 (1.7)	0.02
Jaundice	Phước Long	35	17.8 (2.1)	73	19.8 (2.0)	0.53
	HTD	49	36.2 (2.0)	29	28.4 (2.5)	0.02
	Đồng Xoài	13	26.4 (4.1)	111	18.8 (1.5)	0.10
Cerebral malaria	Phước Long	54	19.4 (2.2)	54	19.0 (2.1)	0.91
	HTD	44	34.3 (2.1)	34	32.1 (2.6)	0.50
	Đồng Xoài	44	22.6 (2.6)	80	17.9 (1.7)	0.11
Haemoglobinuria	Phước Long	14	21.2 (4.1)	94	18.9 (1.6)	0.61
	HTD	16	26.0 (3.3)	62	35.2 (1.8)	0.02
	Đồng Xoài	15	21.3 (4.4)	109	19.3 (1.5)	0.65
Bleeding	Phước Long	2	25.5 (5.5)	106	19.1 (1.5)	0.57
	HTD	5	35.8 (5.8)	73	33.2 (1.7)	0.69
	Đồng Xoài	4	21.5 (11.1)	120	19.5 (1.4)	0.80
Renal failure	HTD	18	34.2 (2.7)	59	32.8 (2.0)	0.73
	Đồng Xoài	6	38 (9.3)	118	18.6 (1.4)	0.003
Respiratory distress	Phước Long	2	43.0 (12.0)	106	18.7 (1.5)	0.03
	HTD	19	34.8 (2.6)	59	32.8 (2.0)	0.60
	Đồng Xoài	9	28.9 (7.9)	115	18.8 (1.4)	0.07
Hyperparasitaemia	Phước Long	41	16.1 (2.4)	67	21.1 (1.9)	0.10
	HTD	18	30.5 (2.9)	60	34.2 (1.9)	0.34
	Đồng Xoài	47	17.6 (2.4)	77	20.8 (1.8)	0.28
Shock	HTD	2	45.5 (4.5)	76	33.0 (1.7)	0.23
Acidosis	HTD	4	40.25 (5.8)	74	33.0 (1.7)	0.33
Hyperlactataemia	Phước Long	9	28.4 (7.1)	99	18.3 (1.5)	0.06
	HTD	14	41.6 (3.9)	64	31.5 (1.7)	0.02
	Đồng Xoài	4	22.8 (6.5)	120	19.5 (1.5)	0.69

Table 7.11: Age associations by centre

Syndrome	Number of cases with syndrome	Mean (SEM) age of cases with syndrome	Number of cases without syndrome	Mean (SEM) age of cases without syndrome	p value
Severe anaemia	23	29.8 (2.3)	170	33.0 (1.0)	0.27
Jaundice	75	34.0 (1.6)	118	31.8 (1.2)	0.26
Cerebral malaria	96	33.7 (1.4)	97	31.6 (1.3)	0.28
Haemoglobinuria	33	29.4 (2.1)	160	33.3 (1.1)	0.12
Bleeding	8	35.5 (4.8)	185	32.5 (1.0)	0.54
Renal failure	23	36.5 (2.8)	169	32.0 (1.0)	0.13
Respiratory distress	27	37.0 (2.5)	166	31.9 (1.0)	0.07
Hyperparasitaemia	53	32.2 (1.8)	140	32.8 (1.1)	0.78
Shock	2	45.5 (4.5)	191	32.5 (1.0)	0.17
Acidosis	4	40.3 (5.8)	189	32.5 (1.0)	0.25
Hyperlactataemia	23	39.3 (3.0)	170	31.7 (1.0)	0.01

Table 7.12. Age syndrome relationships amongst all adults in study

Syndrome	Number of cases with syndrome	Mean (SEM) age of cases with syndrome	Number of cases without syndrome	Mean (SEM) age of cases without syndrome	p value
Severe anaemia	24	6.1 (1.0)	93	7.0 (0.4)	0.39
Jaundice	22	8.8 (1.1)	95	6.4 (0.4)	0.02
Cerebral malaria	46	6.9 (0.6)	71	6.8 (0.5)	0.89
Haemoglobinuria	12	5.2 (1.0)	105	7.0 (0.4)	0.18
Bleeding	3	10.7 (3.4)	114	6.7 (0.4)	0.12
Respiratory distress	3	3.0 (1.0)	114	6.9 (0.4)	0.13
Hyperparasitaemia	53	6.2 (0.5)	64	7.4 (0.6)	0.14
Hyperlactataemia	4	6.0 (2.8)	113	6.8 (0.4)	0.71

Table 7.13. Age syndrome relationships amongst all children in case control study

Ethnic group variation

Sufficient data were available for comparison between Kinh and S'tiếng ethnic groups. Initial analysis suggests that severe anaemia was significantly more common amongst the S'tiếng and the higher incidence of jaundice in the Kinh and hyperparasitaemia in the S'tiếng almost reach significance (Table 25 appendix 2). No S'tiếng patients were recruited at HTD, however, and given the centre dependent clinical profile of cases, further analyses were conducted using only data from cases recruited at Phước Long and Đồng Xoài. Whilst the ethnic differences in jaundice and hyperparasitaemia disappeared, the effect on anaemia remained (25% of S'tiếng vs 14% of the Kinh), although no longer significant (p=0.076) (Table 7.14). This association would be of considerable interest, if real, given the high prevalence of haemoglobinopathies amongst the S'tiếng. Two obvious potential confounders are centre and age. The same number of S'tiếng cases were recruited at Đồng Xoài and Phước Long. More Kinh were recruited at Đồng Xoài, but this would act to dilute the association found as Đồng Xoài cases had a higher prevalence of anaemia. Đồng Xoài cases were older and included fewer young children (Table 7.2). S'tiếng cases were younger than the Kinh, but there was no significant difference in age between anaemic and non-anaemic cases amongst the Kinh (19.3 vs 24.3 p=0.16) or S'tiếng (10.1 vs 6.6 p=0.17). Multiple logistic regression of anaemia on age, centre and ethnic group (or any derived binary or, in the case of age, categorical variables) revealed no significant associations. Whilst this suggests that the apparent association between

being S'tiếng and presenting with anaemia may be spurious, it remains sufficiently intriguing to merit further analysis with the genotype results.

Syndrome	Percentage of Kinh with syndrome	Percentage of S'tiếng with syndrome	p value
Anaemia	14	25	0.08
Jaundice	21	21	1.00
Cerebral	44	38	0.45
Haemoglobinuria	13	13	0.95
Bleeding	4	0	0.16
Renal failure	2	2	0.98
Respiratory distress	4	4	0.98
Hyperparasitaemia	35	44	0.23
Shock	0	2	0.08
Hyperlactataemia	5	6	0.83
Total number	160	52	

Table 7.14. Syndrome categories in Kinh and S'tiếng ethnic groups (Phước Long and Đồng Xoài only)

Severity

A higher proportion of cases at HTD met the more stringent severity criteria, which was unsurprising given its position as a tertiary referral centre (table 7.15). There was no difference in proportions of cases meeting the more stringent criteria between Phước Long and Đồng Xoài, or between S'tiếng and Kinh at Phước Long or Đồng Xoài or both combined (table 7.16).

Centre	Percentage of cases meeting more severe criteria
HTD	70.5%*
Đồng Xoài	47.2%
Phước Long	48.6%

Table 7.15. Severity by centre (*p=0.001 vs other two centres)

Centre	Ethnic group	Number of cases	Percentage meeting more severe criteria	p value
Phước Long	Kinh	75	51%	0.82
	S'tiếng	26	46%	
Đồng Xoài	Kinh	79	51%	0.28
	S'tiếng	26	39%	
Phước Long and Đồng Xoài	Kinh	154	51%	0.30
	S'tiếng	52	42%	

Table 7.16. Difference in proportion of more severe cases between ethnic groups

Controls

Community controls

A total of 630 community controls were recruited. Excluding cases with insufficient criteria, and the 40 cases with missing case samples, 254 cases have 2 community controls and 8 have only 1. Twenty cases have no community controls, although at the time of writing, 15 of these were pending in the control gathering process.

The matching process was not always successful. Ten controls for eight cases were of the opposite sex, and sex data was not recorded for 2 cases and 4 controls. The complete community control set for 4 cases consisted of individuals of the opposite sex or whose sex had not been recorded. None of these cases or controls were excluded. Age was not recorded for 8 cases and 11 controls. 312 controls for 214 cases were outside the age ranges specified. Using a slightly more relaxed set of criteria (+/- 1 year in the under 5's, +/- 2 years from 5 to 15, +/- 5 years from 16 to 25 and +/- 10 years thereafter) brought 140 controls within range. Just over half of the remaining 170 poorly matched controls were under 10 years of age. It is difficult to estimate what effect, if any, this will have on the final results. Age matching was introduced for three reasons: to include an element of matching for malaria risk, as age was second only to ethnic group as a predictor of parasitaemia in the initial survey; to exclude differential mortality rates associated with HbE as a confounder; and to exclude any unknown age-related confounders. Survey data on the relationship between age and smear positivity is depicted in fig 7.2 for S'tiếng and fig 7.3 for Kinh. Appropriate matching groups for the S'tiếng based on this data, knowledge of historical epidemiology of the region and assumptions about exposure, immunity and risk of severe disease would appear to be <3,3-11,12-22, 23-48, 49-67 and 68+. The relationship between age and parasitaemia is less evident amongst the Kinh, but equivalent bands might be <4, 4-15, 16-25, 26-50, and 51+. There does not appear to be any important relationship between age and prevalence of HbE in either Kinh or S'tiếng

(figs 7 & 8 appendix 2). The relationship between unknown confounders and age cannot, of course, be predicted, but experience with other illnesses, and our knowledge of sociological factors, would suggest that categorisation into infants, under five's, school age children, younger adults, middle aged adults and older adults would be appropriate. Using bands of <3, 3-5, 6-13, 14-24, 25-49, 50-65 and >65, with increasing flexibility at the margins of the bands with increasing mean band age still results in 124 mismatched controls for 92 cases. Once again just over half of these cases are under 10. Given the lack of association between age and prevalence of HbE, however, none of these cases have been excluded.

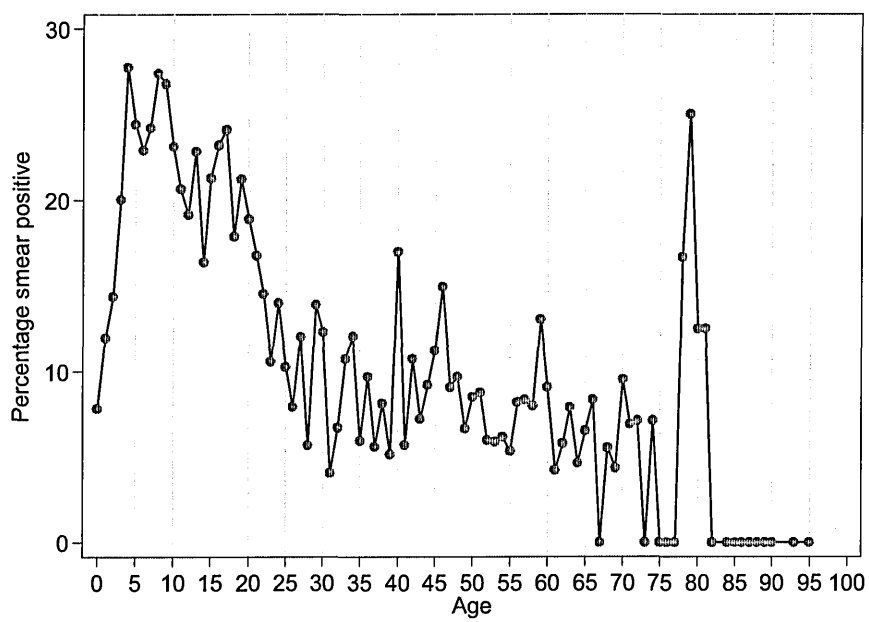


Fig 7.2. Relationship between age and smear positive prevalence amongst S'tiêng survey subjects (age is integer age, i.e. 0≡0-<1).

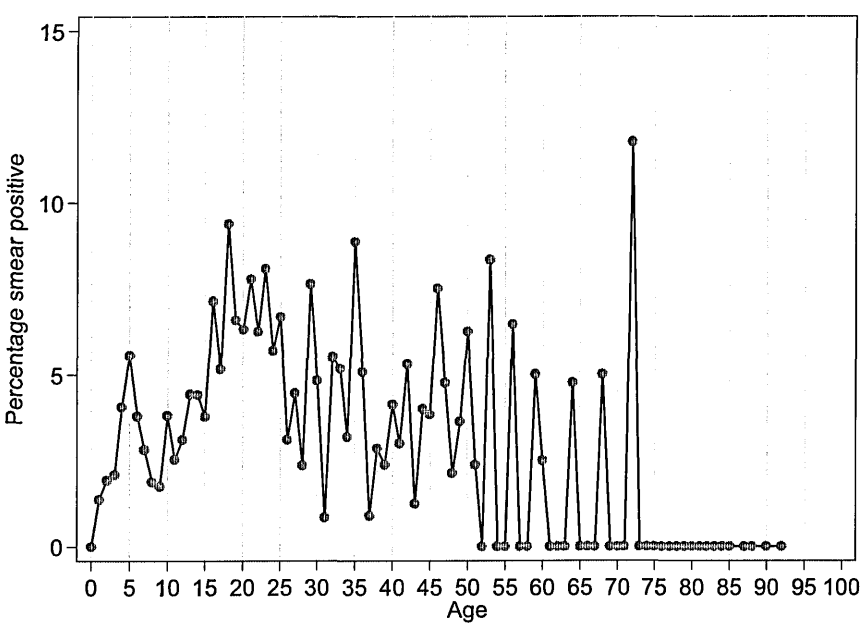


Fig 7.3. Relationship between age and smear positive prevalence amongst Kinh survey subjects.

Family controls

A total of 365 family controls were recruited. 133 cases have 2 family controls each, a further 73 have one, and only one case has 3 family controls. There were 73 case and parent triplets. After excluding ineligible or missing cases, 115 cases have 2 family controls, 69 cases have one, and there are 60 family triplets.

Haemoglobinopathy prevalence

Haemoglobin E

The overall prevalence of haemoglobin E in cases and community controls is given in table 7.17, with ethnic group breakdown in table 7.18. Three points are immediately apparent: the number of cases likely to be informative is small; HbE appears to be *more* prevalent in cases than controls; and there are fewer HbE heterozygotes amongst the controls than would be expected from the extensive cross sectional survey data presented in chapter 3. Although this does not quite reach significance (35% vs 44% p=0.06), it is worth considering whether there may be a potential source of bias here. The large number of observations in the surveys will have given a good estimate of the population prevalence, the only concern being the lack of data on relationships between subjects in the first survey. This would not bias the HbE prevalence estimates unless individuals from families with a high burden of HbE were more likely to attend the survey than those without (within an ethnic group). This might be the case if, for instance, the morbidity (and possible resultant economic disadvantage) from chronic, low-grade anaemia (from HbE) resulted in these families being poorer, or suffering more health problems, and thus being more likely to attend for a free medical

Status	Normal	Haemoglobin E heterozygotes	Haemoglobin E homozygotes
Controls	421 (87%)	49 (10%)	15 (3%)
Cases	204 (85%)	30 (12%)	7 (3%)

Table 7.17. HbE amongst cases & community controls

Ethnic group	Status	Normal	HbE heterozygote	HbE homozygote
Kinh	Controls	344 (96%)	12 (3%)	1 (<1%)
	Cases	176 (96%)	5 (3%)	2 (1%)
S'tiêng	Controls	49 (51%)	34 (35%)	14 (14%)
	Cases	13 (33%)	23 (59%)	3 (8%)

Table 7.18. HbE amongst cases and controls in the two major ethnic groups (minor ethnic groups not shown)

examination. The evidence we have suggest this is not the case, however: the estimate of HbE prevalence in Đắc Ơ from the first survey is no different to that in subsequent surveys which utilised more rigorous sampling strategies (carrier prevalence 65.0% vs 62.8%, gene frequency 0.411 vs 0.407). The initial surveys could not exclude a degree of population stratification with regard to HbE. The crude HbE gene frequencies for different villages are given in table 7.19. Đắc Ơ seems to have consistently higher prevalence than other communes, and there is variation amongst the S'tiếng, but there is no predictable geographic pattern: the two communes immediately adjacent to Đắc Ơ (Đức Hạnh and Đak Nhau) have the lowest prevalence, whilst Đồng Tâm, which lies 70km south of Đắc Ơ has the second highest prevalence. The S'tiếng cases are drawn from 13 different communes, and numbers are too small to make meaningful comparisons with the survey data. Đức Hạnh was the only survey commune to contribute significant numbers of S'tiếng community controls (33), amongst whom the E gene frequency was 0.258, however this is not significantly lower than the 0.305 found in the surveys. There does not appear to be any definite evidence or plausible mechanism for suggesting that the community controls are unrepresentative of the population.

District	Commune	Ethnic group	E gene frequency	p value vs entire sample	p value vs opposite extreme commune
Đồng Phú	Đồng Tâm	Kinh	0.019	1.00	0.27
Bù Đăng	Dak Nhau		0.017	0.89	0.29
Phước Long	Đa Kia		0.014	0.02	0.002
	Đắc Ơ		0.024	**0.01**	**0.002**
	Đức Hạnh		0.018	0.86	0.13
Overall			0.018		
Đồng Phú	Đồng Tâm	S'tiếng	0.376	0.54	0.08
Bù Đăng	Dak Nhau		0.298	0.08	0.002
Phước Long	Đa Kia		0.357	0.67	<0.001
	Đắc Ơ		0.413	**<0.001**	**0.002**
	Đức Hạnh		0.305	**<0.001**	**<0.001**
Overall			0.350		

Table 7.19. Variation in prevalence of HbE between communes. p values generated by comparing allele numbers between commune sample and a) the remainder of the sample from the same ethnic group or b) the commune with the highest prevalence, for communes with lower than average prevalence, or that with the lowest prevalence, for communes with higher than average prevalence.

Alpha thalassaemia

Genotyping for α thalassaemia was a tremendously resource intensive process. The need to maximise genetic material from a case control study led to exploration of techniques for whole genome amplification (WGA). Initial attempts at WGA with random primers (primer extension preamplification – PEP) was unsuccessful: the PEP PCR product would not amplify in the $\alpha^{3.7}$ PCR. Multiple displacement amplification (MDA) WGA was just entering commercial availability at that stage, but negotiations over the acquisition of the system led to further delays, compounded by the need to run MDA and subsequent PCR's in 96 well plates (and thus wait until they are filled). The study (and its funding) had finished by the time everything was in place to conduct the alpha thalassaemia genotyping. Therefore we elected to restrict the genotyping to the S'tiếng, for two reasons: the need to examine an interaction between α thalassaemia and HbE, and the low prevalence of α thalassaemia amongst the other ethnic groups precluding the possibility of finding a significant effect of α thalassaemia in those groups. Table 7.20 details the prevalence of the 3 α thalassaemia mutations examined in S'tiếng cases and community controls.

Mutation	Case status	Normal	Heterozygote	Homozygote	Number
α^{SEA}	Cases	97%	3%	N/A	34 (2)
	Controls	92%	8%	N/A	51 (17)
	Survey	94%	6%	N/A	890 (93)
$\alpha^{3.7}$	Cases	59%	41%	0	32 (4)
	Controls	60%	34%	6%	65 (3)
	Survey	65%	27%	8%	948 (35)
α^{CS}	Cases	97%	3%	0	34 (2)
	Controls	98%	2%	0	52 (16)
	Survey	94%	5%	1%	969 (14)

Table 7.20. Prevalence of α thalassaemia mutations in case control study. Percentages of valid genotypes for that mutation listed. Data from S'tiếng survey subjects only included for comparison. Number column contains number of valid genotypes (missing genotypes). Missing may reflect failed MDA or failed genotype PCR. Totals are lower than tables 7.18 and 7.19 as no controls were genotyped where case sample missing.

The prevalence of $\alpha^{3.7}$ is slightly higher (gene frequency 0.231 vs 0.215, p=0.12) and that of α^{CS} significantly lower than that found in the cross sectional surveys (gene frequency 0.010 vs 0.035 p=0.03). Although statistically significant, the dearth of α^{CS} is minimal in absolute numbers: 3 or 4 heterozygotes would have been expected on the basis of the survey frequencies. It is once again clear that there are no dramatic differences between cases and controls (although perhaps a hint that α^{SEA} heterozygotes and $\alpha^{3.7}$ homozygotes may be protected), but numbers are small.

Relationship between malaria and haemoglobinopathies

Haemoglobin E

The collection of more than one control does not allow for the simple tabulation of case control relationships. Some intuitive sense of the data can be obtained by tabulating case genotype against average E gene frequency amongst the controls. Table 7.21 contain such a summary of the data, from which it is clear that the vast majority of the case control sets contain no member with HbE.

There would appear to be more sets above the "diagonal" of parity between case and average control genotype than below it, suggesting that HbE is not protecting against disease. Conditional logistic regression analysis demonstrated no significant protection afforded by carriage of haemoglobin E, whether heterozygote, homozygote, or, in a separate analysis, both considered together (table 7.22). Restricting the dataset to S'tiếng cases yields a subsample with a higher proportion of informative sets (table 7.23), and the effects of

	Case genotype		
Average E gene freq in controls	AA	AE	EE
0	146	**8**	**2**
0.5	*9*	**9**	**2**
1	*5*	5	**0**
1.5	*2*	*1*	**0**
2	*1*	*0*	1

Table 7.21: Average E gene frequency amongst matched controls by case genotype. Shaded boxes mark the diagonal division between case control sets indicating a protective effect of HbE (in italics), and those suggesting susceptibility (in bold).

haemoglobin E begin to reach significance (table 7.24). Far from the expected protective effect, however, HbE, in particular heterozygous HbE, seems to predispose to severe malaria as defined in this study. As mentioned in the introduction, the severity criteria were deliberately lax, with the intention of examining the subset of cases meeting more stringent criteria separately. Tables 7.25 and 7.26 present the case genotype vs average control gene frequency for HbE for all such cases, and such cases in S'tiếng, respectively.

Genotype	Number of cases	Number of cases with discordant controls	Odds ratio	Standard error	95% confidence interval		p value
AE	241	43	1.7	0.58	0.87	3.30	0.12
EE			1.1	0.56	0.41	2.98	0.85
Any E	241	43	1.7	0.54	0.92	3.19	0.09

Table 7.22: Conditional logistic regression of case status on heterozygous and homozygous HbE, and, separately, on E carrier status

Genotype	Number of cases	Number of cases with discordant controls	Odds ratio	Standard error	95% confidence interval		p value
AE	39	27	3.6	1.83	1.34	9.76	0.01
EE			0.6	0.44	0.15	2.48	0.49
Any E	39	27	2.6	1.18	1.09	6.31	0.03

Table 7.23. Conditional logistic regression of case status on heterozygous and homozygous HbE, and, separately, on E carrier status, for S'tiếng cases only.

	Case genotype		
Average E gene freq in controls	AA	AE	EE
0	3	**5**	**2**
0.5	*0*	**9**	**0**
1	*3*	5	**0**
1.5	*2*	*1*	**0**
2	*1*	*0*	1

Table 7.24. Average E gene frequency amongst matched controls against case genotype for S'tiếng cases only.

Conditional logistic regression analysis of these subgroups are presented in table 7.28. These results are a more difficult to interpret, not least because the number of informative cases becomes very small. The susceptibility attributable to heterozygous HbE appears to

be much stronger for moderate rather than severe malaria, although the odds ratio remains firmly towards the susceptibility side for the S'tiếng. There is a non-significant suggestion that homozygous HbE is protective against more severe disease. The surprising direction of the effect coupled with the apparent dearth of heterozygotes amongst the community controls prompted us to compare the cases with unselected community controls from the cross sectional surveys, stratifying by ethnic group. These analyses were also conducted both for all cases and for those meeting the more stringent criteria (table 7.27). The comparisons with unmatched controls are compatible with the matched analysis, in that most groups of cases carry slightly more HbE than would be expected from the community prevalence, with the exception of the more severe S'tiếng cases. The differences are small, however, and all fail to even approach significance.

	Case genotype		
Average E gene freq in controls	AA	AE	EE
0	82	**3**	**0**
0.5	*8*	**4**	**1**
1	*3*	2	**0**
1.5	*1*	*0*	**0**
2	*1*	*0*	1

Table 7.25. Average E gene frequency amongst matched controls against case genotype for cases meeting more stringent criteria only

	Case genotype		
Average E gene freq in controls	AA	AE	EE
0	1	**1**	**0**
0.5	*0*	**4**	**0**
1	*1*	2	**0**
1.5	*1*	*0*	**0**
2	*1*	*0*	1

Table 7.26. Average E gene frequency amongst matched controls against case genotype for S'tiếng cases meeting more stringent criteria only

The analysis of the family controls is similarly limited by the small number of informative pedigrees. There were only 18 informative transmissions in the 60 family triads, in which E was transmitted 5 times and A 13 (p=0.0593). This runs contrary to the community control data and suggests that E may be exerting a protective effect, although once again this fails to reach statistical significance.

Subgroup	AA	AE	EE	Gene frequency	p value
Kinh community prevalence	96%	4%	0	0.019	
Kinh cases	176 (96%)	5 (3%)	2 (1%)	0.025	0.44
Kinh less severe cases	74 (95%)	3 (4%)	1 (1%)	0.032	0.24
Kinh more severe cases	100 (97%)	2 (2%)	1 (1%)	0.019	1.00
S'tiêng community prevalence	42%	45%	13%	0.357	
S'tiêng cases	13 (33%)	23 (59%)	3 (8%)	0.372	0.81
S'tiêng less severe cases	6 (27%)	14 (64%)	2 (9%)	0.409	0.53
S'tiêng more severe cases	7 (41%)	9 (53%)	1 (6%)	0.324	0.86

Table 7.27. Comparison of cases with population prevalence of HbE measured during the cross sectional surveys. p value from chi squared comparison of allele numbers.

Subgroup	Genotype	Number of cases	Number of cases with discordant controls	Odds ratio	Standard error	95% confidence interval		p value
All	AE	241	43	1.7	0.6	0.9	3.3	0.12
	EE			1.1	0.6	0.4	3.0	0.85
	Any E	241	43	1.7	0.5	0.9	3.2	0.09
More severe	AE	129	23	1.0	0.5	0.4	2.5	0.98
	EE			0.6	0.4	0.1	2.5	0.46
	Any E	129	23	0.9	0.4	0.4	2.0	0.75
Less severe	AE	107	20	**4.6**	2.8	1.3	15.5	**0.02**
	EE			2.5	1.9	0.6	10.9	0.22
	Any E	107	20	**5.3**	3.0	1.7	16.4	**0.004**
S'tiêng only	AE	39	27	**3.6**	1.8	1.3	9.8	**0.01**
	EE			0.6	0.4	0.2	2.5	0.49
	Any E	39	27	**2.6**	1.2	1.1	6.3	**0.03**
More severe S'tiêng	AE	17	10	3.1	2.3	0.7	13.4	0.12
	EE			0.3	0.3	0.03	2.4	0.23
	Any E	17	10	1.5	1.0	0.4	5.7	0.54
Less severe S'tiêng	AE	22	17	**4.1**	2.9	1.1	16.0	**0.04**
	EE			1.3	1.1	0.2	7.1	0.80
	Any E	22	17	3.8	2.3	1.2	12.6	0.03

Table 7.28. Conditional logistic regression of case status on heterozygous and homozygous HbE, and, separately, on E carrier status, for severity and ethnic group subgroups, with whole group results repeated for comparison.

Alpha thalassaemia

The analysis of the impact of α thalassaemia on severe malaria is complicated by the presence of four α globin genes, and the existence of 3 polymorphic mutations in this population. Although α^{CS} appears to have more clinical impact than $\alpha^{3.7}$, it nevertheless has significantly less impact on α globin chain production than α^{SEA}, and has therefore

been grouped with $\alpha^{3.7}$ as disrupting one α globin gene as opposed to the two of α^{SEA}.

Table 7.29 details the relationship between case and control genotypes by number of intact α globin genes. All tables in this section relate to S'tiếng only, as they are the only ethnic group in the case control study to have been genotyped for the α thalassaemia mutations. Numbers of informative case control sets are, once again, small, but do appear to suggest some protection from α thalassaemia. This is borne out by the conditional logistic regression analysis (table 7.30), but the effect fails to reach significance.

		Case genotype (number of intact α globin genes)			
		1	2	3	4
Average number of intact α globin genes amongst controls	2	**0**	0	*1*	*0*
	2.5	**0**	**0**	*0*	*3*
	3	**1**	**2**	5	*7*
	3.5	**0**	**1**	**4**	*10*
	4	**1**	**1**	**2**	7

Table 7.29. Depiction of α globin genotypes in cases and their matched controls. Cell contents are numbers of case control sets. Numbers in bold indicate sets favouring, and those in bold sets opposing, a protective effect of α thalassaemia

Number of α genes deleted	Number of cases	Number of cases with discordant controls	Odds ratio	Standard error	95% confidence interval		p value
One	50	35	0.7	0.33	0.29	1.79	0.47
Two			0.4	0.33	0.07	2.02	0.26
Any	50	35	0.8	0.33	0.36	1.78	0.59

Table 7.30. Conditional logistic regression of case status on one and two gene deletion α thalassaemia, and, separately, on α thalassaemia status.

Restricting these analyses to cases meeting the more stringent severity criteria made little difference to the results (tables 7.31 and 7.32), if anything reducing the size of any effect, although the numbers are vanishingly small.

Number of α genes deleted	Number of cases	Number of cases with discordant controls	Odds ratio	Standard error	95% confidence interval		p value
One	14	35	1.0	0.78	0.20	4.71	1.0
Two			0.5	0.58	0.04	5.34	0.54
Any	14	35	1.3	0.90	0.34	5.04	0.69

Table 7.31. Conditional logistic regression of case status on one and two gene deletion α thalassaemia, and, separately, on α thalassaemia status. Analysis restricted to cases meeting more stringent severity criteria.

		Case genotype (number of intact α globin genes)			
		1	2	3	4
Average number of intact α globin genes amongst controls	2	**0**	0	*0*	*0*
	2.5	**0**	**0**	*0*	*1*
	3	**0**	**1**	3	*0*
	3.5	**0**	**0**	**2**	*3*
	4	**1**	**0**	**0**	3

Table 7.32. Depiction of α globin genotypes in cases and their matched controls for cases meeting the more stringent severity criteria. Cell contents are numbers of case control sets. Numbers in bold indicate sets favouring, and those in bold sets opposing, a protective effect of α thalassaemia

A simple comparison of prevalence of α thalassaemia amongst cases and controls confirms the insignificantly increased prevalence of α thalassaemia in controls (tables 7.33 & 7.34). The prevalence of α thalassaemia states in the cross sectional surveys is given for comparison. Restricting the analysis to cases meeting the more severe criteria appears to lessen any disparity between cases and controls in the prevalence of α thalassaemia, cases actually having an insignificantly higher prevalence of single gene deletion α thalassaemia than their controls, although the same prevalence as all controls. The observation that both subjects with HbH disease are cases is interesting. Malaria is a severe disease in homozygous sickle cell and β thalassaemic individuals, despite the inability of their red cells to support parasite multiplication in vitro. The baseline haematological indices and physiological status of individuals with HbH disease are much less compromised than those of sufferers of either thalassaemia major or sickle cell disease, but it is not

implausible to postulate that parasitisation of the grossly abnormal HbH erythrocytes might result in severe disease. There is little clinical data to support this, however, as the studies of the relationship of α thalassaemia and malaria have been performed in areas where two gene deletions are rare, and thus HbH does not exist (Allen et al. 1997; Mockenhaupt et al. 2004). Haemolytic crises associated with febrile illness are a known manifestation of HbH disease, thus might lead to inclusion as a case on the basis of severe anaemia. Only one of the two cases fell into this category, however, the other meeting the cerebral malaria inclusion criterion. If individuals with HbH disease were more prone to severe malarial disease, however, this might negatively bias the analysis of the impact of α thalassaemia mutations on malaria. Excluding these two cases did not significantly affect the results of the conditional logistic regression analysis of case status on the presence of one gene, two gene, or any α thalassaemia mutations.

Subject group	Number of functioning alpha globin genes				Total
	1	2	3	4	
Surveys	1%	16%	28%	56%	983
Controls	0	12%	36%	52%	73
Cases	4%	9%	27%	60%	45
Total	2%	11%	32%	55%	118

Table 7.33. Alpha globin genotypes in cases, community controls, and S'tiếng survey subjects.

Subject group	Number of functioning alpha globin genes				Total
	1	2	3	4	
Survey subjects	1%	16%	28%	56%	983
All community controls	0	12%	36%	52%	73
Controls for severe cases	0	13%	27%	60%	45
Cases	6%	6%	38%	50%	16
Total	2%	11%	30%	57%	61

Table 7.34. Alpha globin genotypes in cases meeting more stringent severity criteria only, their community controls, all community controls, and S'tiếng survey subjects.

Small numbers again hamper the genetic case control analysis, with only 14 informative transmissions. The increased number of alleles reduces the power still further. The transmission disequilibrium test results are given in table 7.35. None of these results are

significant, although the relative susceptibility of normal alpha globin genes comes close. The direction of the effect for all α thalassaemia alleles is consistent however, both with the community control data and with one another.

Allele	Observed number of transmissions	Expected number of transmissions	p value
α normal	10	6.5	0.05
α^{SEA}	1	1.5	0.56
$\alpha^{3.7}$	3	5	0.21
α^{CS}	0	1	0.16

Table 7.35. Transmission of the prevalent α thalassaemia alleles to cases. p values represent χ^2 comparison of observed and expected number of transmissions of that allele from parents to cases

Interaction between HbE and α thalassaemia

If the protective effect of HbE against malaria were mediated via globin chain imbalance as a result of the mild β thalassaemia consequences of the mutation, it might be expected that coinheritance of α thalassaemia would reduce or abolish this effect. The recent report of negative epistasis between α thalassaemia and HbS with regard to the malaria protection phenotype (Williams et al. 2005) suggests that such an interaction is possible even if the protective effect of HbE is mediated by other mechanisms than globin chain imbalance. Tables 7.36-7.39 display the relationship between HbE and α thalassaemia genotypes of cases and controls in the presence and absence of α thalassaemia and HbE respectively.

	Case genotype		
Average E gene freq in controls	AA	AE	EE
0	4	**2**	**1**
0.5	*1*	**0**	**0**
1	*0*	3	**0**
1.5	*0*	*2*	**0**
2	*2*	*0*	0

Cases and controls with α thalassaemia

	Case genotype	
AA	AE	EE
1	**3**	**1**
0	**2**	**0**
2	3	**0**
1	*0*	**0**
2	*1*	0

Subjects without α thalassaemia

Tables 7.36 & 7.37. Relationships between case and control Haemoglobin E genotype for cases and controls with (7.36) and without (7.37) coinherited α thalassaemia. Case control sets with complete discordance for α thalassaemia are therefore excluded.

		Case genotype (number of intact α globin genes)			
		1	2	3	4
Average number of intact α globin genes amongst controls	2	**0**	0	*1*	*1*
	2.5	**0**	**0**	*0*	*1*
	3	**0**	**0**	3	*4*
	3.5	**0**	**1**	**0**	*0*
	4	**1**	**0**	**2**	6

Cases and controls with Haemoglobin E

	Case genotype (number of intact α globin genes)			
	1	2	3	4
2	**0**	1	*0*	*1*
2.5	**0**	**0**	*0*	*0*
3	**1**	**1**	2	*3*
3.5	**0**	**0**	**0**	*0*
4	**0**	**0**	**0**	2

Subjects without Haemoglobin E

Tables 7.38 & 7.39. Relationships between case and control α thalassaemia genotype for cases and controls with (7.38) and without (7.39) coinherited Haemoglobin E. Case control sets with complete discordance for Haemoglobin E are therefore excluded.

It is clear both that the numbers of informative case control sets are, once again, vanishingly small, and that, nonetheless, there is no obvious gross epistatic effect by which the apparent susceptibility of HbE heterozygotes can be explained. Repeating the exercise examining the interaction of HbE and α thalassaemia in cases alone (i.e. including all controls for those cases regardless of coinheritance) did not alter the patterns seen (tables 26-29, appendix 2). There are clearly insufficient numbers to examine this interaction for cases meeting only more stringent severity criteria. Conditional logistic regression analysis of case status on both haemoglobin E and α thalassaemia status in their different permutations are displayed in tables 7.40 and 7.41, with the unadjusted odds ratios recapitulated for reference. Including both HbE and α thalassaemia in the models only alters individual effects where the full genotypes are involved: a slight diminution of the non-significant protection afforded by two gene deletion α thalassaemia is apparent, and heterozygous haemoglobin E, far from becoming less of a risk factor for severe malaria, as might be expected if its putative protective effect was being negated by coinheritance of α thalassaemia, becomes an even more potent susceptibility factor, an effect which now reaches significance.

Genotype	Odds Ratio	Standard error	95% confidence interval		p value
Univariate analyses					
Any haemoglobin E	1.1	0.32	0.63	1.94	0.72
Any α thalassaemia	0.7	0.25	0.33	1.38	0.29
Multivariate analysis					
Any haemoglobin E	1.2	0.46	0.58	2.53	0.61
Any α thalassaemia	0.6	0.24	0.31	1.33	0.23

Table 7.40. Multivariate conditional logistic regression of case status on presence of any haemoglobin E and α thalassaemia, with univariate analyses for reference.

Genotype	Odds Ratio	Standard error	95% confidence interval		p value
Separate multivariate analyses					
Heterozygous HbE	1.4	0.43	0.76	2.54	0.28
Homozygous HbE	0.3	0.20	0.11	1.07	0.07
One non-functional α gene	0.5	0.21	0.23	1.16	0.11
Two non-functional α genes	0.5	0.32	0.15	1.76	0.29
Single multivariate analysis					
Heterozygous HbE	2.7	1.31	1.02	6.98	0.05
Homozygous HbE	0.3	0.22	0.05	1.33	0.11
One non-functional α gene	0.5	0.23	0.22	1.25	0.15
Two non-functional α genes	0.8	0.55	0.20	3.10	0.72

Table 7.41. Multivariate conditional logistic regression analysis of case status on haemoglobin E and α thalassaemia genotype, with separate HbE and α thal genotype analyses shown for reference.

The number of S'tiếng families with complete HbE and α thalassaemia genotypes is insufficient for multilocus allele transmission analysis, which is therefore unavailable to confirm or refute the community control study findings.

Discussion

The striking result from this study is the absence of a protective effect of haemoglobin E, with the suggestion that heterozygotes may even be predisposed to moderate, and possibly even severe, disease. The major limitation of the study is the small numbers of informative case control pairs (or families, in the case of genetic controls).

This study failed to reach the planned sample size. Three factors conspired to prevent sufficient recruitment: the delayed initiation of recruitment at Đồng Xoài probably lost around 100 cases; the proportion of S'tiếng cases was lower than projected (17 vs 30%),

thus limiting the number of informative case-control sets; and the incidence of severe malaria declined dramatically over the 4 years of the study. The anticipated proportion of S'tiếng cases was probably an overestimate. It was based on the proportion of S'tiếng in the population (extrapolated from data for Phước Long district to the whole of Bình Phước province, which in retrospect was incorrect), the higher burden of malaria amongst the S'tiếng in the cross sectional surveys, and the (limited) admission data from Phước Long hospital. In fact, 30% of the cases recruited at Phước Long *were* S'tiếng, but they comprised only 17% of Đồng Xoài cases, and none of those from HTD. Three possible reasons for this are the age distribution of severe malaria in the S'tiếng, a lower incidence of severe manifestations of clinical disease amongst the S'tiếng, and ethnic referral bias. Almost all S'tiếng cases were children, and would thus be less likely to have been referred to HTĐ if they required tertiary care (the one child case with renal failure was a 2 year old S'tiếng girl who was referred to Paediatric Hospital No. 1 for her renal replacement therapy). There appeared to be no difference in severity between Kinh and S'tiếng cases in Phước Long and Đồng Xoài, as measured by proportions meeting the more stringent severity criteria, or by a crude count of the number of entry criteria met (tables 7.15 and 7.42). Efforts to expand the study to other sites serving HbE rich populations bore fruit too slowly to avoid the dramatic decline in severe malaria incidence.

Centre	Ethnic group	Number of cases	Mean no criteria met (SE)	p value
Phước Long	Kinh	80	1.46 (0.099)	0.0186
	S'tiếng	26	1.92 (0.146)	
Đồng Xoài	Kinh	80	1.39 (0.092)	0.2537
	S'tiếng	26	1.19 (0.096)	
Phước Long and Đồng Xoài	Kinh	160	1.43 (0.067)	0.3131
	S'tiếng	52	1.56 (0.100)	

Table 7.42. Ethnic differences in crude criteria count score at Phước Long and Đồng Xoài.

The small number of informative cases restricts the conclusions that can be drawn from this data. The susceptibility of HbE carriers, in particular heterozygotes, is statistically significant, however, and cannot be dismissed out of hand. This is a completely

unexpected outcome, which flies in the face of previous data, and requires both a close post hoc re-examination of study design and conduct for potential sources of bias, and a re-evaluation of earlier studies, before speculating on the significance of this result.

The only methodological issues affecting case recruitment are inappropriate recruitment and under-ascertainment. It is conceivable that recruiting doctors at the remote hospitals may have embellished the severity of patient's illness if they felt that study entry might be financially beneficial to patient or doctor (inconceivable at HTD). The vast majority of study notes at Đồng Xoài were cross checked against hospital notes by Dr Thái, however, who also witnessed a significant proportion of the study patients first hand. The study notes at Phước Long were designed to capture detailed data about cases, and any apparent discrepancies or important missing data was checked against the hospital notes. No inappropriately recruited cases were discovered through this confirmatory process: all patients subsequently excluded for not meeting entry criteria were clearly identified from either the study notes or the proper enumeration of parasite densities. Complete ascertainment of cases arising in a population is necessary for comparison with controls drawn at random from that population. The recruitment of matched controls renders this issue irrelevant. Even considering the comparison between cases and the cross sectional survey subjects, it is difficult to imagine a set of circumstances in which under-ascertainment of cases would result in such a positive bias. Problems with the selection and recruitment of community controls are much more likely to result in bias. Overmatching is always a concern with a matched case control study design. Any bias generated by overmatching would expect to be negative, however, as overmatched controls are more similar to their cases for study variables than their unmatched counterparts. The exclusion of individuals who have spent 48 hours or more in hospital for a non-traumatic, non-surgical reason might bias the control group if HbE was a risk factor for hospital admission. One early Thai study reported a higher prevalence of HbE amongst 414 patients than 592 "normal" controls (17% vs 11%, p=0.006) (Na-Nakorn et al. 1956). The patients

were drawn from wards and outpatient clinics of a Bangkok hospital, from a Bangkok maternal and child health clinic, and from samples taken by a malaria control team. The authors do not describe what proportion of patients were being treated for anaemia or haematological disorders. The normal controls were drawn from medical and nursing staff of the hospital, and medical students. The authors excluded individuals from families known to harbour HbE (with no comment as to whether "normal" families were included, or whether the family history of HbE was established more frequently in patients or controls). The disparity between patients and controls was overwhelmingly in the pure Thai women (27% vs 13%). It is difficult to comment further on the significance of this result without more data on the subjects. A Malaysian prevalence survey found a non-significantly lower prevalence among 167 inpatients than 369 laboratory workers and blood donors (1.8% vs 3.6%, p=0.23) (Lie-Injo et al. 1972). Individuals admitted for haematological reasons were excluded. All subjects were Malay. A further Thai study reported a prevalence of 18.9% (N=211) in community surveys compared to 20.6% in febrile individuals admitted to the local hospital, which were not significantly different (Kruatrachue et al. 1961). None of these studies distinguished between HbE heterozygotes and homozygotes. There are no prospective studies examining risk of illness associated with HbE. The experience during the early part of this study, when controls were accidentally recruited who did not fulfil this criterion, would suggest that even if HbE were to have an association with hospitalisation, it would have a limited impact: no more than 10% of controls needed to be replaced. The study protocol envisaged community controls being selected from the nearest house to that of a case in a direction selected at random. It was further stipulated that the controls should be unrelated to the case and to each other. Most of the control collection teams operated unsupervised after initial training. There is a suspicion that rather than follow the protocol, the teams would question the case's household as to the nearest house which might contain individuals of a suitable age to be controls. This might lead to an increased chance that controls were related to cases, albeit

not closely. Alternatively, households might protect friends and family from the possibility of blood letting by deliberately not informing the study team of certain households. The tendency for relatives to live in close proximity to one another might have resulted in controls being somehow related to cases even if control houses were chosen according to the protocol. Even if controls were more related to cases than envisaged in the study design, blood relationships between cases and controls would result in a negative bias. If controls were related to one another, but not cases, that might result in a positive bias. There was no correlation between the genotypes of the two controls, however, and repeating the analysis with only one of the community controls yielded essentially the same results (tables 30-32, appendix 2).

All bar one of the potential systematic biases would appear, therefore to be negative biases, and cannot explain away the finding that heterozygous HbE appears to be a susceptibility factor for severe malaria. If there is an association between carrying HbE and risk of all cause hospitalisation, that might go some way to explaining the lower prevalence of heterozygous HbE amongst community controls than cross sectional survey subjects (not statistically significant, but not negligible), although an element of population stratification cannot be ruled out, as discussed above. Whilst it's impossible to exclude a biased underestimate of HbE amongst community controls, as we do not have local data on HbE and hospitalisation, it should be reiterated that the striking feature of this data set is the high prevalence of HbE amongst cases, rather than the low prevalence amongst controls, although the latter may contribute to the matched analysis reaching significance.

In the absence of an obvious bias to account for the counter-intuitive result, a detailed search for confounders is in order. The small number of informative cases limit the possibilities for including potential confounders in multiple regression models, but there is some value in examining these variables in the larger datasets. Factors known to affect the chance of suffering severe malaria should be considered, including malaria transmission

intensity, malaria exposure related behaviour, bednet use, healthcare seeking behaviour, pre-immunity and age. In order to confound there must be a relationship between HbE and the confounding variable, in addition to that with (severe) malaria. That relationship may be systematic, or accidental (ie due to a very skewed distribution of one or more of these variables between subjects with and without HbE occurring by chance due to small numbers). The most important potential confounder in this study is ethnic group, which may also confound the relationship between HbE and any potential confounder. Thus any discussion of data in the ensuing paragraphs refers to ethically adjusted data, and, in most instances (except where explicitly noted), to S'tiếng only, as the other ethnic groups have insufficient HbE to document associations.

Whilst the presence of malaria resistance traits may lead to populations remaining in high prevalence areas, it seems less likely that this relationship pertains over the unstable microepidemiological variations in an endemic area, particularly in an ethnic group displaying limited mobility. The only way to conclusively document the absence of this association would be to conduct an unfeasibly large, comprehensive and detailed combined entomological and haemoglobinopathy prevalence study.

Malaria risk behaviour might be related to HbE if HbE modulated malaria symptomatology. There is no suggestion from the cross sectional data that this is the case (table 5.29, Chapter 5). Exploring potential relationships between HbE and healthcare seeking behaviour is more complicated. The first survey dataset differed from that of subsequent surveys, apart from the documentation of the receipt of any treatment in the two weeks prior to the survey. There was no difference in the proportion of subjects receiving treatment in the preceding fortnight between those with and without HbE. There was a tendency against using the local market as a source of medical treatment in S'tiếng subjects with HbE in the second and subsequent surveys (11% vs 18%, p=0.01). There was no association between HbE and buying medicine directly from the market amongst

S'tiếng subjects in the first survey, however, although it is unclear whether this refutes the association, or reflects the different questions posed. It is difficult to imagine a biologically plausible explanation for this association, but if it were to be a real phenomenon, the outcome would be difficult to predict: treatment obtained in this fashion would be inferior to that prescribed at the hospital or health station, which would be a complete course based on a more accurate diagnosis, rather than a couple of doses of antipyretic, a couple of doses of antibiotic, and a couple of doses of an antimalarial (which might be chloroquine, or might be an artemisinin derivative) which is the usual market prescription. On the other hand, purchasing medicine directly might lead to more rapid initiation of treatment, and a lower threshold for treatment, than making the journey to the health station or hospital, with their relatively restrictive opening hours. An attempt was made in the first survey to assess the timing of a response to illness in the family. Completion rates were very poor, but it appears that individuals with HbE were more likely to wait at home for 1-2 days to see if there was any improvement, rather than either waiting to get sick, or going to the health station immediately (table 7.43). No differences in healthcare seeking behaviour with HbE were found in the case control study community controls, but the numbers here were very small. All the healthcare seeking behaviour questions were plagued by poor completion rates, casting doubt on all of these associations.

Action taken in case of fever	AA	AE	EE	Total number
Wait one or two days to see if fever relents	24%	44%	32%	25
Wait until ill before going to health station	36%	50%	14%	22
Go straight to health station	44%	44%	13%	16
No answer	47%	41%	12%	76

Table 7.43: Healthcare seeking patterns in S'tiếng subjects in the first survey. p value for waiting for improvement vs others for all HbE = 0.04.

Bednet use is such a strong risk factor that despite there being no reason to suppose a relationship between HbE and bednet use, particularly in view of the lack of association between HbE and symptomatology, this possibility should be explicitly discounted. There was a slight tendency for bednet use to increase with HbE carriage in the cross sectional surveys (table 7.44).

Bednet user?	AA	AE	EE	Any HbE	Total number
Yes	45%	42%	13%	55%	3055
No	50%	40%	10%	50%	333

Table 7.44. Bednet use and HbE. χ^2 any HbE vs no HbE, p=0.08, Cuzick non-parametric test for trend across HbE genotype groups, p=0.04.

No relationship exists between HbE and age (table 7.45). If there is a relationship between HbE and pre-immunity, this would not be considered a confounder, rather an integral part of the effect of HbE on the risk of severe malaria.

Ethnicity	Genotype	N	Mean age +/- σ
All	AA	8620	22.2 +/- 19.0
	AE	2373	22.1 +/- 19.7
	EE	651	23.4 +/- 20.5
S'tiếng	AA	2038	21.7 +/- 19.7
	AE	1934	22.8 +/- 19.9
	EE	608	23.2 +/- 20.2

Table 7.45: Relationship between HbE and age. p values in all ethnic groups and S'tiếng respectively: 0.14 & 0.11AA vs EE, 0.88 & 0.11 AA vs AE, 0.16 & 0.64 AE vs EE.

The above consideration of potential confounders is mostly relevant to comparisons between cases and unselected community controls (the survey data): matching controls for ethnic group, location, age and sex should dramatically reduce differences in the malaria risk variables between cases and controls. Not only is ethnicity the only documented confounder, but it is likely to influence many aspects of behaviour, including malaria exposure related behaviour, healthcare seeking behaviour, and other less well documented risk factors such as poverty and house construction. Geographic matching should minimise the impact of healthcare accessibility and local variations in malaria transmission.

Matching by age should reduce the risk of confounding by age, but not eliminate it, as the age matching was imperfect: control tended to be older than cases, particularly for younger cases (Fig 7.4). Heterozygous cases appeared slightly older than both normal and homozygous cases amongst the S'tiêng, but there was no difference among controls (table 7.46). Case control sets in which HbE appeared to act as a susceptibility trait were actually significantly better age matched than those in which it appeared to protect (mean case-control age difference –0.9+/-3.5SD years vs –4.2+/-6.8, p=0.01).

The tendency of the S'tiêng to remain close to their family home (none of the S'tiêng case families described themselves as migrant) means the age and location matching are probably reasonable proxies for previous malaria exposure in this ethnic subgroup, which is an important determinant of pre-immunity. The absence of migration might therefore lead to a more predictable relationship between age and pre-immunity amongst the S'tiêng. The location matching might also reduce variation in behavioural factors, as families living adjacent to one another may behave in a similar fashion, although there is no guarantee that they might not differ in important ways.

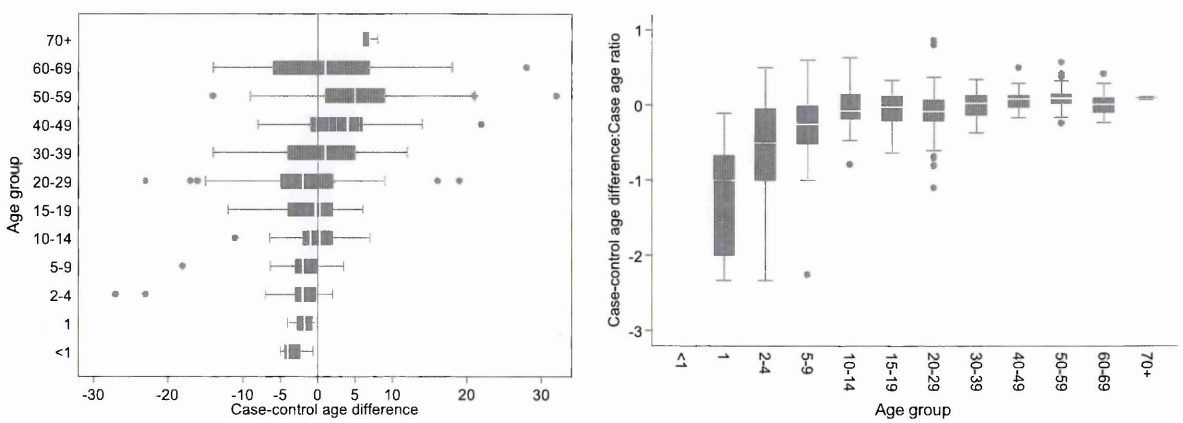


Fig 7.4. Difference between age of case and ages of community controls by case age. Differences are displayed as absolute values (left hand graph) and relative to age of case (right hand graph). The two outliers in the 2-4 age group are omitted in the right hand graph.

Hb type	Cases				Community controls			
	N	Mean age ± σ	p values		N	Mean age ± σ	p values	
AA	11	3.9+/-2.0	0.03	0.10	38	6.4+/-4.5	0.64	0.07
AE	20	6.7+/-3.7			24	6.9+/-3.5		
EE	3	2.8+/-1.3	0.43		12	9.9+/-8.8	0.15	

Table 7.46. Relationship between age and genotype in S'tiêng cases and community controls.

The small numbers limit the formal examination of potential confounders in the case control data, as mentioned above, but it may be reassuring to examine the relationship between the unmatched (mostly behavioural) variables and HbE in the cases and community controls. Somewhat surprisingly the community controls had a very similar rate of health care contact in the preceding fortnight. This was much higher than the 18% (15% amongst S'tiêng) found in the cross sectional surveys. There appeared to be a higher rate of health care contact amongst S'tiêng homozygote cases and controls, although this could not be statistically significant in the cases, where there are only three homozygotes (table 7.47). There were no differences in choice of healthcare provider between cases and controls, or by HbE genotype. Seventeen percent (9/54) of S'tiêng cases admitted not regularly using bednets in the home, compared to 3% of matched controls (3/115). The distribution of HbE amongst the 7 non-bednet using cases with valid HbE genotypes did not differ significantly from population frequencies, however, one case being homozygous for HbE (14%), 3 heterozygous (42%) and 3 normal (42%). Data on other high risk behaviours, such as forest exposure, were not gathered.

HbE status	Cases		Controls	
	N	Treatment in last 2 weeks	N	Treatment in last 2 weeks
AA	16	63%	45	51%
AE	26	58%	35	51%
EE	3	100%	19	89%
Total	45	62%	99	59%

Table 7.47. Treatment seeking in 2 weeks prior to study contact in S'tiêng cases and community controls. Control homozygotes vs other genotypes $p<0.02$.

Alpha thalassaemia is known to occur at high frequencies, and G6PD deficiency at moderate frequencies, amongst the S'tiêng. Once again there would still need to be an

association between HbE and α thalassaemia or G6PD deficiency for either to act as a confounder. A systematic association is not possible, as the three traits are carried on different chromosomes. An accidental association of HbE with a protective trait could only explain an apparent protective effect, however, and not a susceptibility, although the interaction with α thalassaemia is complicated by the theoretical possibility of negative epistasis between α thalassaemia and HbE with regard to protection from severe malaria. This might reduce the apparent protective effect of HbE even in the absence of a very skewed distribution of α thalassaemia. Were epistasis to be complete, however, it would still not result in an apparent susceptibility of HbE carriers. The number of informative case control sets is too small to formally examine epistasis, as conditional logistic regression models including interaction terms between HbE and α thalassaemia fail to converge. Comparing the odds ratios for HbE terms in models including both HbE and α thalassaemia does not suggest a diminution of a protective effect of HbE by coinheritance of α thalassaemia (if anything, the odds ratio for HbE increases – tables 7.40 and 7.41). The same pattern is seen in conditional logistic regression models stratified by the presence of α thalassaemia (tables 7.48 and 7.49).

Genotype	Odds Ratio	Standard error	95% confidence interval		p value
Case does not have α thalassaemia mutation					
Heterozygous HbE	2.7	1.60	0.84	8.63	0.10
Homozygous HbE	0.2	0.23	0.02	1.79	0.15
Any HbE	1.3	0.63	0.53	3.36	0.54
Case has α thalassaemia mutation					
Heterozygous HbE	4.5	4.04	0.78	26.1	0.09
Homozygous HbE	2.1	2.79	0.15	28.9	0.59
Any HbE	3.9	3.31	0.74	20.5	0.11

Table 7.48. Conditional logistic regression analysis, on S'tiêng data only, of case status on haemoglobin E genotype, and, separately, presence of any haemoglobin E, stratified by the presence of α thalassaemia in the case.

Genotype	Odds Ratio	Standard error	95% confidence interval		p value
Case and control(s) do not have α thalassaemia mutation					
Heterozygous HbE	2.7	2.24	0.50	13.9	0.25
Homozygous HbE	0.3	0.34	0.03	2.71	0.28
Any HbE	1.1	0.71	0.34	3.84	0.84
Case and control(s) have α thalassaemia mutation					
Heterozygous HbE	4.6	5.84	0.39	54.8	0.22
Homozygous HbE	1.9	3.00	0.09	41.4	0.68
Any HbE	3.6	4.25	0.37	35.8	0.27

Table 7.49. Conditional logistic regression analysis, on S'tiếng data only, of case status on haemoglobin E genotype, and, separately, presence of any haemoglobin E, stratified by the presence of α thalassaemia in cases & controls.

The family association study did not confirm the result of the matched control study, suggesting instead that the β^E allele was protective. Thus whilst neither detectable bias nor confounding nor epistasis with α thalassaemia appeared to explain the apparent susceptibility of HbE heterozygotes, it is not insignificant that the TDT, which is immune to such considerations, gives the opposite result to the matched population control analysis. The TDT is prey to concerns over non-paternity and differential fetal survival rates, however, with the former being of particular concern given the widespread documentation of 10-30% non-paternity in similar environments. The very small number of informative transmissions also casts doubt on the validity of this result. It is not therefore possible to state with any degree of certainty that the matched control analysis has produced a spurious result due to the influence of an unsuspected and unmeasured confounder. Conflicting results from large well run family association and case control studies in the same populations have been reported recently (Wilson et al. 2005), with associations found in community control studies not being corroborated by the family analysis, and vice versa, but there were no instances of the two techniques appearing to show associations in opposite directions.

In view of the conflict with published results, a reappraisal of the robustness of the literature is essential, in particular the four clinical studies examining the relationship between HbE and clinical malaria discussed in chapter 1. The first Thai case control study

was a poor quality retrospective study of 122 children admitted with malaria, which found no difference in the incidence of severe disease or death between children with HbE and those without (Kruatrachue et al. 1970). The definition and documentation issues with this study have been outlined in Chapter 1. Despite the small sample size and methodological weaknesses, however, there is no obvious bias unless clinical syndrome recording accuracy was associated with HbE genotype. The later Thai hospital based case control study of acute falciparum malarial in eastern Thailand failed to show an effect of HbE (Kitayaporn et al. 1992). Recruitment was limited to hospital attendees that the authors felt were epidemiologically at risk of malaria, defined as being between 2 and 60 years old and living in one of 21 out of 35 villages served by the district hospital. Cases were patients with acute or severe falciparum malaria, frequency matched by sex and age (in 3 broad bands) to controls selected from patients whose blood films were negative (and were thus likely to have had some symptom or sign compatible with a diagnosis of malaria, such as fever or anaemia). Slightly fewer cases than controls had HbE (AE 32.1% vs 37.6%, EE 6.6% vs 4.8% N=271 each group), but this effect was not significant. The authors also questioned participants about fava bean consumption in the month prior to recruitment, and constructed a logistic regression model of being a case on haemoglobin type, previous malaria history, bed net use, fava bean consumption, and the interactions between homozygous and heterozygous HbE and fava bean consumption. The latter was a significant protective effect (OR 0.26 , p=0.032) as was having suffered the previous attack of malaria greater than 4 months ago (OR 0.65, p=0.031). This study has the advantages of being prospective and having adequate numbers of subjects with HbE, but the disadvantages of using hospital controls chosen on the basis of factors (eg anaemia) which might be associated with HbE (although usually only homozygous E), and of relying on subjects recall of dietary habits for its significant outcome. The Burmese case control study also failed to show any effect of HbE on the proportion of patients admitted with acute malaria to suffer severe disease (Oo et al. 1995). The 383 subjects in this study were

all adults (slightly unusual in an environment which still has high malaria transmission) from the majority ethnic group, and were recruited at one of two hospitals. It is not entirely clear whether recruitment was prospective or not. The authors describe the cases as being "recruited on an individual basis", and it appears clear from the laboratory methods that certain investigations, such as assays of G6PD deficiency, which one would not expect to be routine in that environment, were carried out on freshly drawn blood. On the other hand, the study is described as a "survey", and refers to subjects being "selected" (although it is possible that this refers to the exclusion of children, ethnic minorities, and individuals who had received malaria treatment in the previous 7 days). It seems most likely that subjects were prospectively recruited. All subjects were included in the haemoglobinopathy analysis, regardless of G6PD status. The effects of G6PD status and combinations of haemoglobinopathy and G6PD deficiency were presented in detail separately. The authors categorised subjects as having α thalassaemia trait without adequate DNA analysis, but all categories of haemoglobinopathy were reported, allowing the reader to reanalyse the data, combining normal subjects with those inadequately diagnosed as having α thalassaemia trait. On this basis 14% of 86 HbE carriers had severe disease by the strictest criteria, compared to 13% of normal subjects (and 2% of β thalassaemia carriers). Excluding the subjects putatively diagnosed as carrying α thalassaemia raised the proportion in normal subjects to 15%, the same proportion as HbE heterozygotes considered alone (there being only 8 HbE homozygotes in the sample). The categorisation of severity was slightly unusual, as the authors distinguished subjects with hyperparasitaemia (>5%) alone, mild to moderate impairment of consciousness (GCS 9-14), and all other severe manifestations from uncomplicated acute malaria. Once again, however, all data were reported, allowing the reader to reanalyse the data as appropriate, although it was not possible to distinguish those with GCS between 9 and 11 from those with a GCS between 12 and 14. No permutation of analysis results in a significant protective effect of HbE. The only study to demonstrate a significant protective effect of

HbE was conducted at the Hospital for Tropical Diseases in Bangkok(Hutagalung et al. 1999). The authors *retrospectively* analysed 318 individuals admitted with malaria who happened to have had haemoglobin typing requested during their admission. The proportion of HbE heterozygotes admitted with severe malaria was compared with that amongst individuals with normal haemoglobin, and found to be over 6 times lower. The authors had excluded all individuals with G6PD deficiency, or those in whom G6PD status had not been determined. G6PD activity had been assayed acutely, however, which can be unreliable in individuals acutely unwell with conditions, such as malaria, associated with haemolysis, as the higher proportion of younger red cells (with higher G6PD activity) found in such situations can lead to false negatives. All individuals with β globin abnormalities other than heterozygous HbE were also excluded, as were all individuals with a low MCV and no detectable haemoglobinopathy, in order to eliminate those with α thalassaemia. There are two problems with this latter strategy: many individuals with single gene deletion are not microcytic, and will thus be included, and this manoeuvre will have reduced the frequency of α thalassaemia amongst the population without HbE, whilst leaving that amongst the HbE heterozygotes unchanged. There were 62 such subjects in a total of 101 exclusions on the basis of haemoglobin type or G6PD status. The authors report the ethnic origins of their subjects, and take these into account during the analysis. It is not clear whether ethnicity was derived from different naming conventions between the ethnic groups, or impressive and exemplary recording of ethnicity in case notes or on request forms. Amongst the 72 Thai (a fairly heterogeneous group), a third carried HbE and 4 (8%) had severe disease, whilst amongst 112 Mon, 13% carried HbE and 20% had severe disease, the proportions amongst 33 subjects of other ethnicities being 9% and 21% respectively. The authors thus quite appropriately stratified their analysis by ethnic group. Only 1 HbE carrier had been admitted with severe disease, although this individual was also the only subject to die. The odds ratio for subjects without HbE having been admitted

with severe rather than acute malaria was 6.9, but the confidence interval was very wide (1.2-146.4), and this result was only just statistically significant (p=0.044).

Thus the one study which has shown a protective effect of HbE against severe malaria is significantly methodologically flawed, and the magnitude of the effect is unfeasible in view of the data presented here and the weaknesses of the study design. No previous study, however, has suggested that HbE carriers might be more susceptible to severe malaria, and this outcome is biologically implausible. Susceptibility to severe malaria would apply a strong negative selective pressure, and any allele conferring susceptibility would rapidly be lost. The criteria for severe malaria in this study are such that it is possible that not all cases would have died in the pretreatment era, but the morbidity would also have been a significant negative pressure. It is difficult to imagine what other fitness benefits HbE might afford, and virtually unimaginable that these benefits would outweigh both the (relatively small) negative fitness impact of the homozygous state, and a susceptibility to malaria. Similarly, whilst it is not outwith the bounds of possibility that HbE is linked to another beneficial allele, the positive selective pressure associated with that allele would have to be immense to overcome the negative pressure of malaria, and the linkage would have to apply across all areas where HbE has become prevalent. It is possible that HbE might be close to neutral in populations unaffected by malaria, given the mild phenotype of the homozygote, but all populations in which HbE dwell in malarious areas.

It seems most likely that HbE does protect against malaria, and the results in this study reflect the low power and the low protective efficacy of HbE. Whilst α thalassaemia is clearly protective in other studies, it does not consistently appear so in this study, and the protective efficacy of single and two gene deletion α thalassaemia appear equal, contradicting the findings of both previous studies. It is notable that neither study showed a statistically significant protective effect of heterozygous single gene deletion

α thalassaemia, supporting the idea that such protection does not need to be large in the face of a benign homozygous phenotype in order for the allele to become highly prevalent under selection by malaria.

Conclusion

The decline of malaria in the study region prevented the sample size being achieved in this study. The data that were gathered show no protective effect of HbE against moderate or severe malaria, and hint at heterozygotes being at increased risk, although the family association study demonstrates a non-significant effect in the opposite direction. The small number of informative case control sets and genetic transmissions limit the conclusions which can be drawn from this work, but it nevertheless suggests that any protection HbE affords against malaria is small, and calls into question the one previous study to have found a large effect.

Chapter 8 – Conclusion

The aims of this body of work were to document and quantify the protective effect of haemoglobin E against malaria. Secondary aims were to explore potential mechanisms by examining the relationship between HbE and parasitaemia, parasite species, and symptomatology. Detailed information on the local malaria epidemiology was an essential basis for the association studies.

The discovery that the S'tiếng experienced both the highest prevalence of malaria and the highest prevalence of HbE was very exciting: the suggestion that α-thalassaemia might exert its protective effect through "natural immunisation", and the mild thalassaemic phenotype of haemoglobin E combining to generate an anticipation that we might be witnessing the same phenomenon. It was also clear that ethnicity was potentially a major confounder of any effect, and would need to be adjusted for in all analyses. The young age of the S'tiếng cases in the case control study, and the steeper decline of parasite prevalence with age amongst the S'tiếng than other ethnic groups might hint at just such an effect, or might simply reflect greater transmission. The S'tiếng were as symptomatic with parasitaemic episodes as other groups, which might count against an immune mediated effect, although closer analysis of the data suggests that they might be more prone to over-reporting symptoms. The accumulating evidence of a multiplicity of red cell disorders amongst the S'tiếng cast doubt on whether any phenomena apparent at the ethnic level might be due to haemoglobin E, whilst the stable prevalence of HbE across age groups implies a lack of effect on mortality in the past 3 generations.

The archaeological and historical records give little information about the origins of many of the ethnic groups living in the study area, although the haemoglobinopathy genetic data does seem to correspond with linguistic classifications (with the exception of the Malayo-Polynesian speakers). The lack of good data on geographic ranges of the different ethnicities, the movement of some populations to areas of different malaria endemnicity

(eg the migration of the Tày from the relatively malaria free southern Chinese plains to the malarious northern Vietnamese uplands), and the fact that malaria was endemic throughout Vietnam for centuries (albeit with more intense transmission in the highlands) mean that any attempt to construct a microepidemiological association study, such as that of Siniscalco, would be a purely speculative exercise.

The release of individuals' haemoglobin types did not confirm the initial hypothesis. There was no relationship between HbE and parasite prevalence in the cross sectional survey samples. Whilst disappointing, it has rarely been possible to document a relationship between any red cell disorder and malaria in such studies, except in the case of HbS. There was also no effect on parasite count or species. There was no effect of HbE on the symptomaticity of smear positive episodes, or the relationship between symptomaticity and age, which was an unfortunate finding for the natural immunisation theory, although subject to the reporting biases noted above.

The case control study failed to achieve sufficient numbers of cases from populations with a reasonable prevalence of HbE. Amongst the 55 S'tiếng cases recruited there was no evidence of a protective effect, however, with a suggestion that individuals with HbE might even be more susceptible to severe disease. This finding was contradicted by the results from the genetic control study, although numbers were very small. It was not possible to draw any conclusions about differences between heterozygous and homozygous HbE, or differences between moderate and severe disease, as numbers were too few. Two gene α thalassaemia did seem to offer some protection against severe disease, confirming results from other studies. Numbers were to small to examine the hypothesis that the apparent susceptibility of HbE carriers was due to negative epistasis with α thalassaemia, but this is not supported by the distribution of α thalassaemia in cases and controls, and analysing only those case-control sets without α thalassaemia did not reverse the apparent susceptibility associated with HbE.

In the absence of any demonstrable effect of haemoglobin E, the aetiology of the interethnic differences in transmission remained a mystery. The KAP study undertaken to explain this variation produced few convincing findings, although only 90% of S'tiếng households reported regularly using bednets, compared to 97% amongst the Kinh. There were also differences in unproven risk factors, such as wealth and education, but not in other major behavioural risks such as forest activity. Whilst these findings might provide an explanation for the variation in transmission, the absence of any differences in behaviour between the S'tiếng and other ethnic minority groups (which also have a lower transmission rate), casts some doubt on this conclusion. There were insufficient smear positive episodes during the KAP study to document locally relevant risk factors.

All studies were hampered by the reduction in malaria transmission during the period under study. It is unclear whether this decline is a recent phenomenon, or a delayed continuation of the trend initiated in the early 1990's, which appeared to have plateaued in Bình Phước towards the end of the decade. Bednets and artemisinin derivatives have certainly been responsible for much of this achievement, with less certain roles for deforestation and increasing GDP. If the witnessed decline is more recent, it may be due to increasing adoption of artemisinin derivatives in the more remote health stations, or their greater penetration into the informal sector; the additional 1500 bednets distributed in the study area in 2000 and 2001 as part of the province wide EC malaria programme; continuing deforestation, which is likely to increase with the construction of new roads, or the slow "trickling down" of national wealth to the poor rural areas. This last is likely to have exercised its effect, if any, through improved health care infrastructure rather than greater household resources, as most peasant farmers in the region remain poor: temporary gains in income are often dashed, or even reversed, by fluctuations in commodity prices. This study unfortunately provides no data to elucidate the roles of these different factors in the decline in malaria transmission.

Work is ongoing in a number of key areas. An entomological study was set up in the hamlets of Bù Bưng, Thôn 3 and Thôn 7 in Đắc Ơ in 2003, which has yet to report. This study will add to our understanding of factors influencing the microheterogeneity of transmission, but was not established to elucidate the importance of ecological factors in interethnic variation in transmission. It became clear early in the study that assaying case control subjects G6PD activity would not be possible. The mutations responsible for G6PD deficiency in the S'tiếng have not been characterised, and it is possible, given the potentially limited genetic stock, that one or two dominant mutations may be identified, which would thus be amenable to PCR based diagnosis. Preliminary results have, unfortunately, been rather confusing in this regard, and, given the considerable amount of resources necessary to pursue this avenue to its conclusion, this line of enquiry is no longer being pursued. A birth cohort study was always envisaged as an integral part of this project, and has recently completed, but too late for inclusion in the thesis. In addition to better malaria incidence data, this was designed to generate insights into the possible mechanism of action of the protective effect of HbE. As this effect remains to be proven, however, it is unclear whether any useful data will emerge from the cohort study. The window of opportunity has closed for conducting many other interesting additional studies: GIS mapping of the study site is unlikely to add information at this stage, but would have been extremely useful, had permission ever been granted; an early KAP study would have given invaluable information on changes in malaria risk behaviour which might have contributed to the fall in prevalence; and further ecological and entomological studies would be difficult and resource intensive given the extremely low EIR.

There remains plenty of scope for further *in vitro* work on the relationship between haemoglobin E and malaria: the recent demonstration of altered PfEMP1 display on HbC erythrocytes (Fairhurst et al. 2005), and the previously demonstrated differences in antibody binding of thalassaemic erythrocytes (Luzzi et al. 1991a; Luzzi et al. 1991b; Udomsangpetch et al. 1993a; Williams et al. 2002) are both avenues which have not been

pursued for HbE. The interesting observation that heterozygous HbE cells appear more resistant to multiple invasion than homozygous HbE erythrocytes remains to be explained, and whilst the balance of the *in vitro* data has shifted towards demonstrating a protective effect of HbE, differences between studies have yet to be reconciled. A further, comprehensive set of parasite culture experiments in different conditions, with different parasites and repeated many times would go a long way to achieving this.

There is a clear need for a larger case control study in another site with a higher population prevalence of both haemoglobin E and malaria. A major difficulty with such a study is the nature of malaria transmission in Southeast Asia: a certain level of healthcare infrastructure is necessary in order to be able to conduct a good quality, community based case control study. Once such a level of infrastructure is achieved, however, a significant fall in malaria transmission within the ensuing two years or so is an almost inevitable corollary.

In conclusion, this project was unable to demonstrate any protective effect of haemoglobin E against malaria. The extremely high gene frequency of HbE in certain (unrelated) populations remains strongly suggestive of positive selection, and malaria remains the obvious candidate. Whilst these studies were hampered by declining malaria transmission and a relatively low prevalence of HbE in the study population as a whole, they have generated sufficient data to suggest that any protective effect is likely to be small, which may be commensurate with the benign clinical syndromes of HbE in populations devoid of β-thalassaemia resulting in minimal impact on fitness.

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Appendix 1

Study forms

Notes and table of contents

Notes

These study forms have been translated from the Vietnamese originals. Some of the formatting has been unavoidably deranged in the process, and so the forms may not look exactly as the original. Some of the paragraph spacing has been altered in order to preserve reasonable pagination, the KAP family table has lost 2 rows, and the orientation of text in the header row has been changed for certain cells in order to keep the table on one sheet. Otherwise, as few changes as possible have been made so the reader can see how much space, for instance, was available to document the answer to a particular question.

The forms were designed to be used in the field, so do not fit within the page boundaries set down for a thesis. Rather than completely destroy the original layout of the forms, these boundaries have been ignored for this section.

Common pages for the case control study records are not duplicated. Each set of notes consisted of the case sheets, which varied by centre and are reproduced in full, two copies of the parental control sheet, which was the same for all centres, and only one example copy is presented, and two copies of the community control sheet. This was similar in Phước Long and Đồng Xoài, but different at HTD, so one copy of each is included.

Table of contents

Survey Forms

Doctor completing form:_____ Date:_____

Surname:_____ Middle name:_____ First name:_____

Age:____ years ____months Sex:_____ Head of household:_____

Ethnic group:_____ Hamlet:_____

Spleen (cm):_____ Commune:_____

Pregnant? Y / N Months pregnant:___ District:_____

Any treatment in last 2 months? Y / N Other address details:_____

Fever in last 7 days? Y / N District of birth:_____

Fever in last 2 days? Y / N *If under 5 years old*

Temperature:_____ °C Place of birth: At home with TBA Health station
At home, no attendant Hospital

Details of current illness:

If temperature is >37.5°C, please complete all the following questions.

If you have a fever, what would you usually do?

Wouldn't know I had a fever | Wait for 1 or 2 days then go to the health centre | Wait until I was really ill before going to the HC | Buy some medicine straight away | Go to the HC straight away

Symptoms: Rigors? Y / N Chills? Y / N Headache? Y / N Cough? Y / N
Diarrhoea? Y / N More than 5 stools in last 24 hours? Y / N
Sort throat? Y / N Earache/discharge? Y / N Runny nose? Y / N

Signs: Rash/skin lesions? Y / N Details: Viral Exanthem-Specific (eg Measles, VZV)
Viral Exanthem - Non-specific
Jaundice? Y / N Skin Infection - likely cause of fever
Pallor? Y / N Skin Infection - unlikely cause of fever
Abscess
Inflammed ears? Y / N / Couldn't examine Non-infective Dermatitis
Sore throat? Y / N / Couldn't examine Purpura

Resp rate:_____ Abnormal chest sounds? Y / N Details (clearly described):

Crepitations:_____ Bronchial breathing:_____ Rhonchi:_____ Wheeze:_____

Any other signs? Y / N
Details:_____

Diagnosis of cause of fever
URTI
LRTI
Measles
Chickenpox
Probable malaria
Probable dengue
Gastroenteritis
Viral infection
Skin lesions
Other

Other diagnosis:_____

Parasight F result:
Negative
Positive
Not done

Treatment (number of tablets):
Amoxycillin
Artemisinin
Chloramphenicol Eye Drops
Chlorpheniramine
Diclofenac
Maloxal
Multivits (tablets or granules)
Paracetamol
Terpine
Vermifar
Vit C/glucose (box)

Slide taken by:_____ Study number:_____

All questions must be completed

Number

1) Doctor completing form:_____ 2) Date:_____

3) Surname:_____ 4) Middle name:_____ 5) First name:_____

6) Head of household:_____ 7) Group/house number:_____

11) Age:_____ years _____ months 8) Hamlet:_____

12) Sex: Male / Female 9) Commune:_____

12b) Pregnant? Y / N 12c) Months preg:__ 10) District:_____

13) Name of real mother:_____ 14) Ethnic group of real mother:_____

15) Name of real father:_____ 16) Ethnic group of real father:_____

17) Migrant? Y / N ****If the answer to question 17 is "Yes", please complete questions 17b-d****

17b) From which province?:_____ 17c) Which year did you arrive?_____

17d) Why did you move? Government resettlement/Follow spouse/Economic prospects

18) Fever in last 7 days? Y / N 19) Fever in last 2 days? Y / N

20) Rigors? Y / N 21) Chills? Y / N 22) Headache? Y / N

23) Did you receive any treatment in the last 2 weeks? Y / N ****If Yes, please complete Q23b-h****

23b) Reason: Malaria / URTI / Diarrhoea / Fever / Trauma / Chronic illness

23c-h) Where were you treated?

23c) Hospital Y / N 23d) Health station Y / N 23di) Which health station?_____

23e) Bought medicine at the market: Y / N 23f) Private doctor or pharmacist Y / N

23g) Traditional medicine: Y / N 23h) Traditional physical treatments (eg cupping): Y / N

24) Sleep under bednet? Y / N 24b) Use appropriately / use inappropriately

25) Spleen (cm):_____ 26) Temperature:_____°C

26) Slide taken by:_____ 27) Number (please recheck with study number above): _____

28) Treatment: Parasight F result (if performed)

Antimalarials Positive Negative

Other 29) Diagnosis:_____

Follow up

Result of survey slide: Falciparum:_____ Non-falciparum:_____ Falciparum & non-falciparum:___ (complete prior to follow up visit)

Fever since survey? Y / N Rigors since survey? Y / N Chills since survey? Y / N

Headache since survey? Y / N Spleen (cm):_____ Temperature:_____°C

Treatment:_____

All questions must be completed Number

(sticky label)

1) Doctor completing form:_____ 2) Date:_____

3) Surname:_____ 4) Middle name:_____ 5) First name:_____

6) Household number in census:_____

7) Head of household:_____ 8) Head of Group:_____

13) Age:_____ years _____ months 7) Group:_____

14) Sex: Male / Female 10) Hamlet:_____

14b) Pregnant? Y / N 12c) Months preg:__ 11) Commune:_____

12) District:_____

15) Name of real mother:_____ 16) Ethnic group of real mother:_____

17) Name of real father:_____ 18) Ethnic group of real father:_____

19) Migrant? Y / N ****If the answer to question 19 is "Yes", please complete questions 19b-d****

19b) From which province?:_____ 17c) Which year did you arrive?_____

19d) Why did you move? Government resettlement/Follow spouse/Economic prospects

20) Fever in last 7 days? Y / N 21) Fever in last 2 days? Y / N

22) Rigors? Y / N 23) Chills? Y / N 24) Headache? Y / N

25) Did you receive any treatment in the last 2 weeks? Y / N ****If Yes, please complete Q25b-h****

25b) Reason: Malaria / URTI / Diarrhoea / Fever / Trauma / Chronic illness

25c-h) Where were you treated?

25c) Hospital Y / N 25d) Health station Y / N 25di) Which health station?_____

25e) Bought medicine at the market: Y / N 25f) Private doctor or pharmacist Y / N

25g) Traditional medicine: Y / N 25h) Traditional physical treatments (eg cupping): Y / N

26) Sleep under bednet? Y / N 26b) Use appropriately / use inappropriately

27) Spleen (cm):_____ 28) Temperature:_____°C

29) Diagnosis:_____

30) Treatment: Antimalarials ☐ Other ☐

Parasight F test performed? ☐ Result: Positive ☐ Negative ☐

26) Slide taken by:_____ 27) Number (please recheck with study number above): _____

All questions must be completed

Number

1) Doctor completing form:_____ 2) Date:_____

3) Surname:_____ 4) Middle name:_____ 5) First name:_____

6) Year of birth:_____ Month of birth:_____ 8) Sex: Male / Female

7) Ethnic group:_____ 8b) Pregnant? Y / N 8c) Months preg:__

9) Household number in census: ☐ 12) Group:_____

10) Head of household:_____ 13) Hamlet:_____

11) Head of Group:_____ 14) Commune:_____

15) Name of real mother:_____ 16) Ethnic group of real mother:_____

17) Name of real father:_____ 18) Ethnic group of real father:_____

19) Migrant? Y / N ****If the answer to question 19 is "Yes", please complete questions 19b-d****

19b) From which province?:_____ 17c) Which year did you arrive?_____

19d) Why did you move? Government resettlement/Follow spouse/Economic prospects

20) Fever in last 7 days? Y / N 21) Fever in last 2 days? Y / N

22) Rigors? Y / N 23) Chills? Y / N 24) Headache? Y / N

25) Did you receive any treatment in the last 2 weeks? Y / N ****If Yes, please complete Q25b-h****

25b) Reason: Malaria / URTI / Diarrhoea / Fever / Trauma / Chronic illness / Other

25c-h) Where were you treated?

25c) Hospital Y / N 25d) Health station Y / N 25di) Which health station?_____

25e) Bought medicine at the market: Y / N 25f) Private doctor or pharmacist Y / N

25g) Traditional medicine: Y / N

26) Sleep under bednet? Y / N 26b) Use appropriately / use inappropriately

27) Spleen (cm):_____ 28) Temperature:_____°C

29) Diagnosis:_____

30) Treatment: Antimalarials ☐ Other ☐

Parasight F test performed? ☐ Result: Positive ☐ Negative ☐

Sampling details:

Haematocrit:_____%

31) Slide taken by:_____ 32) Number (please recheck with study number above): _____

All questions must be completed

Number (sticky label)

1) Doctor completing form:_____ 2) Date:_____

3) Surname:_____ 4) Middle name:_____ 5) First name:_____

6) Year of birth:_____ Month of birth:_____ 8) Sex: Male / Female

7) Ethnic group:_____ 8b) Pregnant? Y / N 8c) Months preg:__

9) Household number in census: ☐ 12) Group:_____

10) Head of household:_____ 13) Hamlet:_____

11) Head of Group:_____ 14) Commune:_____

15) Name of real mother:_____ 16) Ethnic group of real mother:_____

17) Name of real father:_____ 18) Ethnic group of real father:_____

19) Migrant? Y / N ****If the answer to question 19 is "Yes", please complete questions 19b-d****

19b) From which province?:_____ 17c) Which year did you arrive?_____

19d) Why did you move? Government resettlement/Follow spouse/Economic prospects

20) Fever in last 7 days? Y / N 21) Fever in last 2 days? Y / N

22) Rigors? Y / N 23) Chills? Y / N 24) Headache? Y / N

25) Have you ever had dark brown urine (the colour of coca cola)? Y / N

26) Did you receive any treatment in the last 2 weeks? Y / N ****If Yes, please complete Q26b-h****

26b) Reason: Malaria / URTI / Diarrhoea / Fever / Trauma / Chronic illness / Other

26c-h) Where were you treated?

26c) Hospital Y / N 26d) Health station Y / N 26di) Which health station?_____

26e) Bought medicine at the market: Y / N 26f) Private doctor or pharmacist Y / N

26g) Traditional medicine: Y / N

27) Sleep under bednet? Y / N 27b) Use appropriately / use inappropriately

28) Spleen (cm):_____ 29) Temperature:_____°C

30) Diagnosis:_____

31) Treatment: Antimalarials ☐ Other ☐

Parasight F test performed? ☐ Result: Positive ☐ Negative ☐

Sampling details: Sample kept for G6PD assay? ☐ If so - Haematocrit:_____%

32) Slide taken by:_____ 33) Number (please recheck with study number above): _____

Name:_____

Number (sticky label)

All questions must be completed

1) Doctor completing form:_____ 2) Date:_____

3) Hamlet:_____ 4) Commune:_____

5) Ethnic group:_____

6) Household number in census:_____

7) Individual ID number in census:_____

8) Pregnant? Y / N 8c) Months preg:_____

9) Name of real mother:_____ 10) Ethnic group of real mother:_____

11) Name of real father:_____ 12) Ethnic group of real father:_____

13) Migrant? Y / N ****If the answer to question 13 is "Yes", please complete questions 13b-d****

13b) From which province?:_____ 13c) Which year did you arrive?_____

13d) Why did you move? Government resettlement/Follow spouse/Economic prospects

14) Fever in last 7 days? Y / N 15) Fever in last 2 days? Y / N

16) Rigors? Y / N 17) Chills? Y / N 18) Headache? Y / N

19) Have you ever been admitted to hospital for 48 hours or more? Y / N

19b) If so, why?: Injury / operation / fever / other

20) Have you ever had dark brown urine (the colour of coca cola)? Y / N

21) Did you receive any treatment in the last 2 weeks? Y / N ****If Yes, please complete Q21b-h****

21b) Reason: Malaria / URTI / Diarrhoea / Fever / Trauma / Chronic illness / Other

21c-h) Where were you treated?

21c) Hospital Y / N 21d) Health station Y / N 21di) Which health station?_____

21e) Bought medicine at the market: Y / N 21f) Private doctor or pharmacist Y / N

21g) Traditional medicine: Y / N 21h) Traditional physical treatments (eg cupping): Y / N

22) Did you sleep under a bednet last night? Y / N

23) Spleen (cm):_____ 24) Temperature:_____°C

30) Diagnosis:_____

26) Treatment: Antimalarials ☐ Other ☐

Parasight F test performed? ☐ Result: Positive ☐ Negative ☐

Sampling details:

27) Slide taken by:_____ 28) Number (please recheck with study number above): _____

Name:_____

Number (sticky label)

All questions must be completed

1) Doctor completing form:_____ 2) Date:_____

3) Hamlet:_____ 4) Commune:_____

5) Ethnic group:_____

6) Household number in census:_____

7) Individual ID number in census:_____

8) Pregnant? Y / N 8c) Months preg:_____

9) Name of real mother:_____ 10) Ethnic group of real mother:_____

11) Name of real father:_____ 12) Ethnic group of real father:_____

13) Migrant? Y / N ****If the answer to question 13 is "Yes", please complete questions 13b-d****

13b) From which province?:_____ 13c) Which year did you arrive?_____

13d) Why did you move? Government resettlement/Follow spouse/Economic prospects

14) Fever in last 7 days? Y / N 15) Fever in last 2 days? Y / N

16) Rigors? Y / N 17) Chills? Y / N 18) Headache? Y / N

19) Have you ever been admitted to hospital for 48 hours or more? Y / N

19b) If so, why?: Injury / operation / fever / other

20) The following questions are only for subjects less than 2 years (24 months) old:

Breastfed until what age (time exclusively breast fed only, with no other milk or food) – if never exclusively breastfed, answer 0?:_____months

At what age did the baby commence solid food?:_____months

At what age did the baby stop taking breastmilk altogether?:_____months

21) Did you receive any treatment in the last 2 weeks? Y / N ****If Yes, please complete Q21b-h****

21b) Reason: Malaria / URTI / Diarrhoea / Fever / Trauma / Chronic illness / Other

21c-h) Where were you treated?

21c) Hospital Y / N 21d) Health station Y / N 21di) Which health station?_____

21e) Bought medicine at the market: Y / N 21f) Private doctor or pharmacist Y / N

21g) Traditional medicine: Y / N 21h) Traditional physical treatments (eg cupping): Y / N

22) Did you sleep under a bednet last night? Y / N

23) Spleen (cm):_____ 24) Temperature:_____°C

30) Diagnosis:_____

26) Treatment: Antimalarials ☐ Other ☐

Parasight F test performed? ☐ Result: Positive ☐ Negative ☐

Sampling details:

27) Slide taken by:_____ 28) Number (please recheck with study number above): _____

Name:_____

Number (sticky label)

All questions must be completed

1) Doctor completing form:_____ 2) Date:_____

3) Hamlet:_____ 4) Commune:_____

5) Ethnic group:_____

6) Household number in census:_____

7) Individual ID number in census:_____

8) Pregnant? Y / N 8c) Months preg:_____

9) Name of real mother:_____ 10) Ethnic group of real mother:_____

11) Name of real father:_____ 12) Ethnic group of real father:_____

13) Migrant? Y / N ****If the answer to question 13 is "Yes", please complete questions 13b-d****

13b) From which province?:_____ 13c) Which year did you arrive?_____

13d) Why did you move? Government resettlement/Follow spouse/Economic prospects

14) Fever in last 7 days? Y / N 15) Fever in last 2 days? Y / N

16) Rigors? Y / N 17) Chills? Y / N 18) Headache? Y / N

19) Do you get tired or out of breath easily? Y / N

20) Do you get tired or out of breath on exercise? Y / N

21) Have you ever been admitted to hospital for 49 hours or more? Y / N

21b) If so, why?: Injury / operation / fever / other

22) Did you receive any treatment in the last 2 weeks? Y / N ****If Yes, please complete Q22b-h****

22b) Reason: Malaria / URTI / Diarrhoea / Fever / Trauma / Chronic illness / Other

22c-h) Where were you treated?

22c) Hospital Y / N 22d) Health station Y / N 22di) Which health station?_____

22e) Bought medicine at the market: Y / N 22f) Private doctor or pharmacist Y / N

22g) Traditional medicine: Y / N 22h) Traditional physical treatments (eg cupping): Y / N

23) Did you sleep under a bednet **last night**? Y / N

24) Spleen (cm):_____ 25) Temperature:_____°C

26) Jaundice? Y / N 27) Pallor?: Y / N

28) Diagnosis:_____

29) Treatment: Antimalarials ☐ Other ☐

Parasight F test performed? ☐ Result: Positive ☐ Negative ☐

Sampling details:

30) Slide taken by:_____ 31) Number (please recheck with study number above): _____

Name:_____ Year of birth (age if unknown):_____ Number (sticky label)

All questions must be completed

1) Doctor completing form:_____ 2) Date:_____

3) Hamlet:_____ 4) Commune:_____

5) Ethnic group:_____

6) Household number in census:_____

7) Individual ID number in census:_____

8) Pregnant? Y / N 8c) Months preg:_____

9) Name of real mother:_____ 10) Ethnic group of real mother:_____

11) Name of real father:_____ 12) Ethnic group of real father:_____

13) Migrant? Y / N ****If the answer to question 13 is "Yes", please complete questions 13b-d****

13b) From which province?:_____ 13c) Which year did you arrive?_____

13d) Why did you move? Government resettlement/Follow spouse/Economic prospects

14) Fever in last 7 days? Y / N 15) Fever in last 2 days? Y / N

16) Rigors? Y / N 17) Chills? Y / N 18) Headache? Y / N

19) Have you ever been admitted to hospital for 49 hours or more? Y / N

19b) If so, why?: Injury / operation / fever / other

20) Did you receive any treatment in the last 2 weeks? Y / N ****If Yes, please complete Q20b-h****

20b) Reason: Malaria / URTI / Diarrhoea / Fever / Trauma / Chronic illness / Other

20c-h) Where were you treated?

20c) Hospital Y / N 20d) Health station Y / N 20di) Which health station?_____

20e) Bought medicine at the market: Y / N 20f) Private doctor or pharmacist Y / N

20g) Traditional medicine: Y / N 20h) Traditional physical treatments (eg cupping): Y / N

21) How many bednets does your household own?:_____

22) Did you sleep under a bednet last night? Y / N

23) Spleen (cm):_____ 24) Temperature:_____°C

25) Height (cm):_____ 26) Weight (babies – g, adults – kg):_____

27) Diagnosis:_____

28) Treatment: Antimalarials ☐ Other ☐

Parasight F test performed? ☐ Result: Positive ☐ Negative ☐

Sampling details:

29) Slide taken by:_____ 30) Number (please recheck with study number above): _____

Name:_____ Year of birth (age if unknown):_____ Number (sticky label)

All questions must be completed

1) Doctor completing form:_____ 2) Date:_____

3) Hamlet:_____ 4) Commune:_____

5) Ethnic group:_____

6) Household number in census:_____

7) Individual ID number in census:_____

8) Pregnant? Y / N 8c) Months preg:_____

9) Name of real mother:_____ 10) Ethnic group of real mother:_____

11) Name of real father:_____ 12) Ethnic group of real father:_____

13) Migrant? Y / N ****If the answer to question 13 is "Yes", please complete questions 13b-d****

13b) From which province?:_____ 13c) Which year did you arrive?_____

13d) Why did you move? Government resettlement/Follow spouse/Economic prospects

14) Fever in last 7 days? Y / N 15) Fever in last 2 days? Y / N

16) Rigors? Y / N 17) Chills? Y / N 18) Headache? Y / N

19) Have you ever been admitted to hospital for 49 hours or more? Y / N

19b) If so, why?: Injury / operation / fever / other

20) Did you receive any treatment in the last 2 weeks? Y / N ****If Yes, please complete Q20b-h****

20b) Reason: Malaria / URTI / Diarrhoea / Fever / Trauma / Chronic illness / Other

20c-h) Where were you treated?

20c) Hospital Y / N 20d) Health station Y / N 20di) Which health station?_____

20e) Bought medicine at the market: Y / N 20f) Private doctor or pharmacist Y / N

20g) Traditional medicine: Y / N 20h) Traditional physical treatments (eg cupping): Y / N

21) How many bednets does your household own?:___ 21b) How many people live in your house?:__

22) Did you sleep under a bednet last night? Y / N

23) Spleen (cm):_____ 24) Temperature:_____°C

25) Height (cm):_____ 26) Weight (babies – g, adults – kg):_____

27) Diagnosis:_____

28) Treatment: Antimalarials ☐ Other ☐

Parasight F test performed? ☐ Result: Positive ☐ Negative ☐

Sampling details:

29) Slide taken by:_____ 30) Number (please recheck with study number above): _____

Case control forms

Case form

Study Number:_____ Hospital Number:_____

1) Doctor completing form:_____ 2)Date:_____ AAV number:____

3) Informant: Patient / parent (main carer) / parent (not main carer) /main carer (not parent) / spouse or cohabitee / other relative / other Details of other informant:

All the questions below concern the patient, not the individual giving the information to the doctor (if not the patient)

4) Surname:_____ 5) Middle Name:_____ 6) First Name:_____

8) Age:____yrs ____months

9) Sex: M / F

9b) Pregnant? 9c) Months pregnant:___

14) Name of biological mother:_____ Alive/ dead

15) Maternal ethnic group:_____

16) Name of biological father:_____ Alive/ dead

17) Paternal ethnic group:_____

18) Migrant? Y/N 18b) From where?:_____ 18c) When (Year)?_____

18d) Why? Government resettled / Marriage / Spontaneous economic / Spontaneous other

19) Occupation:_____

20) Do you stay away from your home for work? Y/N

20b) How much time do you spend away from home? __nights/week __weeks/month __months/year

Notes:_____

Address when away from home:

20c) Name of head of household:_____ 20d) Name of Head of Group:_____

20e) Group/House Number:_____ 20h) Provincial town:_____

20f) Hamlet/Street Name:_____ 20i) District:_____

20g) Commune/Ward:_____ 20j) City/Province:_____

20k) Bed net use in working environment? Y/N 20l) Appropriate / Inappropriate

21) Previous Malaria? Y/N 21b) When?:_____

21c) Malaria diagnosed on: Clinical / blood test / don't know

22) Bed net use? Y/N 22b) Appropriate / Inappropriate

Category of Severe Malaria:

Cerebral Malaria Y/N --> CSF WBC:_____ CSF glucose:____ Blood glucose:____ CSF protein:___

Renal Failure Y/N --> Highest creatinine:_____

Severe Anaemia Y/N --> Lowest Hb (g/dl):_____ Lowest Hct (%):_____

Macroscopic haemoglobinuria Y/N --> G6PD result:Normal/Abnormal

Pulmonary oedema Y/N EDTA sample for haemoglobinopathy analysis

Jaundice Y/N --> Highest bilirubin:_____ Blood transfusion before sample? Y/N

Hypoglycaemia Y/N--> Lowest glucose:_____ Duration (hours):____ Number of Units:___

Shock Y/N --> Lowest BP:_____/_____ Duration (hours):_____

Spontaneous bleeding Y/N --> Site of bleeding:_____

Hyperlactataemia Y/N --> Highest Lactate:_____

Acidosis Y/N --> Lowest bicarbonate:_____

Smear taken at: Health station/Hospital Smear taken: Before treatment/after commencing treatment

Falciparum initial smear result only

Trophozoites:_____ Schizonts:_____Gametocytes:_____Parasite Density:_____/400 WBC's

Case form: Outcome

Name:_____ Study Number:_____

Outcome: Discharge home - no problems / discharge home - some persisting problems / sent to another hospital / died / relatives took home when still unwell

If sent to another hospital, which one?_____

Date of discharge/transfer:_____

Plan for follow up: No follow up planned / returning to CTD on date:_____ / follow up at home on date:_____ as part of _____ study

Any other information: _____
_____
_____
_____

Community control form

1) Study Number of Case:_____

2) Doctor completing form:_____ 2)Date:_____

4) Informant: Individual themselves / parent (main carer) / parent (not main carer) /main carer (not parent) / other relative / other Details of other informant:

5) Surname:_____ 6) Middle Name:_____ 7) First Name:_____

8) Head of Household:_____ 11) Group/House Number:_____

9) Age:____years _____months 12) Hamlet/Street name:_____

10) Sex: M / F 13) Commune/Ward:_____

10b) Pregnant? Y/N 14) Provincial town:_____

10c) Months pregnant:_____ 15) District:_____

16) City/Province:_____

17) Biological mother's ethnic group:_____ 18) Biological father's ethnic group:_____

19) Migrant? Y/N 19b) From where?:_____ 19c) When (Year)?_____

19d) Why? Government resettled / Forced other / Marriage / Spontaneous economic / Spontaneous other

20) Occupation:_____

21) Do you stay away from your home for work? Y/N

21b) How much time do you spend away from home?:____nights/wk ____wks/month ____months/yr

Where do you stay when you are working away from home?

21c) Group/House Number:_____21f) Provincial town:_____

21d) Hamlet/Street Name:_____ 21g) District:_____

21e) Commune/Ward:_____ 21h) City/Province:_____

21g) Bed net use in working environment? Y/N 21h) Appropriate / Inappropriate

22) Are you currently ill with fever? Y/N

23) Ever been admitted to hospital for more than 48 hours? Y/N

23b) If so, what for?: Trauma / Surgery / Fever / Other

23c) If other, please give as much detail as possible:_____

24) Previous Malaria? Y/N 24b) When?:

24c) Malaria diagnosed on: Clinical / blood test / don't know

25) Bed net use? Y/N 25b) Appropriate / Inappropriate

26) Height_____m 27) Weight_____kg

28) Suitable to be a control? Y/N 29) Consent obtained? Y/N 30) Specimen taken? Y/N

31) Study number of control:_____

Parental/Sibling Control Form

1) Name of Case:_____ 2) Study number of case:_____

3) Doctor completing form:_____ 4) Date:_____

All the following questions concern the control individual, not the case

5) Surname:_____ 6) Middle Name:_____ 7) First Name:_____

8) Head of Household: 11) Group/House Number:_____

9) Age:____yrs____months 12) Hamlet/Street Name:_____

10) Sex: M/F 13) Commune/Ward:_____

16) Maternal ethnic group:_____ 14) District:_____

17) Paternal ethnic group:_____ 15) City/Province:_____

18) Relationship to case: Biological mother / biological father / full sibling / half sibling / current partner of biological mother / current partner of biological father

19) For siblings only:

19a) Name of biological mother:_____

19b) Name of biological father:_____

20) Consent obtained? Y/N 21) Specimen taken? Y/N

22) Study number of control:_____

I _____ have had the purpose of this study explained to me by the doctors/clinical assistants of _____hospital/health centre, and agree that I/my child_____ will be a control in the study.

Date:_____

Signature:_____

Case form page 1: Demographic Details **Study Number:**_____

1) Doctor completing form:_____ Hospital number:_____

2)Date:_____

3) Informant: Patient / real parent / adoptive parent / carer (but not parent) /
spouse or cohabitee / other relative / other (give details:_____)

All the questions below are about the patient, not about the individual providing the answers (unless that is the patient)

4) Surname:_____ 5) Middle Name:_____ 6) First Name:_____

7) Age:____yrs ____months (months must be recorded if <3 yrs old)

8) Sex: M/F

8b) Pregnant? Y/N 8c) Months pregnant:__

14) Name of biological mother:_____ Alive/ dead

15) Maternal ethnic group:_____

16) Name of biological father:_____ Alive/ dead

17) Paternal ethnic group:_____

9) Name of Head of Group:_____

10) Head of Household:_____

11) Group/House Number:_____

12a) Hamlet/Street Name:_____

12b) Commune/Ward:_____

13a) Provincial town:_____

13b) District:_____

13c) Province/City:_____

District malaria team informed yet? Y/N

18) Migrant? Y/N 18b) From where?:_____ 18c) When (Year)?_____

18d) Why? Government resettled / Marriage / Spontaneous economic / Spontaneous other

19) Occupation:_____

20) Do you stay away from your home for work? Y/N

20b) How much time do you spend away from home? __nights/week __weeks/month __months/year

Notes:_____

Address when away from home:

20c) Name of head of household:_____ 20d) Name of Head of Group:_____

20e) Group/House Number:_____ 20h) Provincial town:_____

20f) Hamlet/Street Name:_____ 20i) District:_____

20g) Commune/Ward:_____ 20j) City/Province:_____

20k) Bed net use in working environment? Y/N 20l) Appropriate / Inappropriate

21) Any treatment in last 2 weeks?: Y/N

21b) What for?: Malaria / RTI / Diarrhoea / Other fever / Injury / Chronic illness

Where did you receive your treatment (multiple answers possible)?

21c) Hospital Y/N 21d) Health Station Y/N 21di) Which one?_____

21e) Bought medicine from the market/pharmacy Y/N 21f) Bought medicine from a private HCW Y/N

21g) Traditional herbal treatment Y/N 21h) Traditional physical treatment Y/N

22) Previous Malaria? Y/N 22b) When?:_____

22c) Malaria diagnosed on: Clinical / blood test / don't know

23) Bed net use? Y/N 23b) Appropriate / Inappropriate

Case form page 2: Symptoms

Name:_____ Case number:_____

24) Admit time:_____ 25) Admit date:_____

26) Admit from: Home / Health Station 26b) Which health station:

Symptoms:

	Duration: Days	Hours	
27) Fever? C/K/KB			
28) Headache? C/K/KB			
29) Cough? C/K/KB			29b) Cough productive? Y / N
30) Difficulty in breathing? C/K/KB			
31) Rapid Breathing? C/K/KB			
32) Chest indrawing? C/K/KB			
33) Deep Breathing? C/K/KB			
34) Poor feeding (infants only) C/K/KB			
35) Altered consciousness? C/K/KB			
36) Seizures? C/K/KB			36b) Number of seizures in last 24 hours:_____
37) Prostration? C/K/KB			
38) Loin Pain? C/K/KB			
39) Dysuria? C/K/KB			
40) Dark urine? C/K/KB			
41) Number of times passed urine in last 24 hours			
42) Jaundice? C/K/KB			
43) Spontaneous bleeding? C/K/KB			43b) Bleeding
44) Vomiting? C/K/KB			
45) Diarrhoea? C/K/KB			45b)Number of stools in last 24 hours:_____
			45c) Blood in stools? C/K
46) Constipation? C/K/KB			
47) Rash? C/K/KB			

Case form page 3: Signs

Name:_____ Study number:_____

Signs: Temperature:_____°C Pulse rate:____bpm Blood pressure:____/____mmHg

Height:_____m Date measured:_____

Weight:____kg Date measured:_____

Hydration Status: Well hydrated / 5% dehydrated / 10% dehydrated / 15% dehdrated

Clinical nutrition status: Well nourished / under nourished / malnourished / marasmic / kwash

Cold peripheries? Y/N Clinical haemodynamic status: Adequate / Impending shock / Shock

Jaundice? Y/N Pallor? Y/N Oedema? Y/N Fontanelle (infants only): Sunken/bulging/closed

Time since last seizure: no seizures / <30min / <1hour / >1hour

Altered consciousness? Y/N Meningism? Y/N Focal neurology? Y/N

Glasgow Coma Score: Total: ____/15 Motor: ___/6 Speech: ____/5 Eye opening: ____/4

Paediatric Coma Score: Total: ___/5 Motor: ___/2 Vocalisation: ___/2 Eye movement: ___/1

Resp rate:_____ Cyanosis? Y/N Kussmaul breathing? Y/N Respiratory distress? Y/N

Abnormal chest signs? Y/N Details: Wheeze: Y/N Rhonchi: Y/N Creps: Y/N Bronchial breathing: Y/N

Spleen (cm):_____ Liver (cm):_____

Rash/Skin lesions? Y/N → Details: Viral Exanthem - Specific (eg Measles, VZV)
Viral Exanthem - Non-specific

Inflammed ears? Y/N/Not seen Abscess
Non-infective Dermatitis

Inflammed throat? Y/N/Not seen Purpura
Other (give details)_____

Other signs? Y/N Details:_____

Case form page 4: Lab results

Name:_____ Study Number:_____

Hct:_____% Hb:_____g/dl RBC:_____/µl

WBC manual:_____/µl N:_____% L:____% E:___% Plt:_____

Blood Sugar (bedside test):_____ Blood sugar (laboratory):_____

Lactate:_____

Date and time of LP: _____/_____/_____ ____:

Lumbar puncture: Done / Not done (too sick) / Not indicated / unsuccessful / bloody tap

Opening pressure:____cm CSF CSF WBC____ CSF L:____ CSF N:_____

CSF glucose:_____

Blood culture taken? ☐ BC result: Positive / Negative / contaminant

BC result details:_____

Date and time of CXR: _____/_____/_____ ____:

CXR result:_____

EDTA sample for haemoglobinopathy analysis? ☐

Smear taken at: Health Station / Hospital

Smear taken: Before treatment / after commencing treatment

Falciparum	☐	Plus Score	Vivax	☐	Plus Score
Trophozoites	☐	☐	Trophozoites	☐	☐
Schizonts	☐	☐	Schizonts	☐	☐
Gametocytes	☐	☐	Gametocytes	☐	☐

Parasite Density:

_____/400 WBC's _____/400 WBC's

_____/1000 RBC's _____/1000 RBC's

Case form page 5: Outcome

Name:_____ Study Number:_____

Outcome: Discharge home - no problems / discharge home - some persisting problems / sent to another hospital / died / relatives took home when still unwell

If sent to another hospital, which one?:_____

Date of discharge/transfer:_____

Final Diagnosis:_____

Discharge checklist:

Height recorded?
Weight recorded?
Precise address?
All laboratory results recorded?
Details completed on patient list in HSCC?
List of family members completed?

Community control form

1) Study Number of Case:_____

2) Doctor completing form:_____ 2)Date:_____

4) Informant: Individual themselves / parent (main carer) / parent (not main carer) /main carer (not parent) / other relative / other Details of other informant:

5) Surname:_____ 6) Middle Name:_____ 7) First Name:_____

8) Head of Household:_____ 11) Group/House Number:_____

9) Age:____years _____months 12) Hamlet/Street name:_____

10) Sex: M / F 13) Commune/Ward:_____

10b) Pregnant? Y/N 14) Provincial town:_____

10c) Months pregnant:_____ 15) District:_____

16) City/Province:_____

17) Biological mother's ethnic group:_____ 18) Biological father's ethnic group:_____

19) Migrant? Y/N 19b) From where?:_____ 19c) When (Year)?_____

19d) Why? Government resettled / Forced other / Marriage / Spontaneous economic / Spontaneous other

20) Occupation:_____

21) Do you stay away from your home for work? Y/N

21b) How much time do you spend away from home?:____nights/wk ____wks/month ____months/yr

Where do you stay when you are working away from home?

21c) Group/House Number:_____ 21f) Provincial town:_____

21d) Hamlet/Street Name:_____ 21g) District:_____

21e) Commune/Ward:_____ 21h) City/Province:_____

21g) Bed net use in working environment? Y/N 21h) Appropriate / Inappropriate

22) Any treatment in last 2 weeks?: Y/N

22b) What for?: Malaria / RTI / Diarrhoea / Other fever / Injury / Chronic illness

Where did you receive your treatment (multiple answers possible)?

22c) Hospital Y/N 22d) Health Station Y/N 22di) Which one?_____

22e) Bought medicine from the market/pharmacyY/N 22f) Bought medicine from a private HCW Y/N

22g) Traditional herbal treatment Y/N 22h) Traditional physical treatment Y/N

23) Ever been admitted to hospital for more than 48 hours? Y/N

23b) If so, what for?: Trauma / Surgery / Fever / Other

23c) If other, please give as much detail as possible:_____

24) Previous Malaria? Y/N 24b) When?:_____

24c) Malaria diagnosed on: Clinical / blood test / don't know

25) Bed net use last night? Y/N

26) Height_____m 27) Weight_____kg

28) Suitable to be a control? Y/N 29) Consent obtained? Y/N 30) Specimen taken? Y/N

31) Study number of control:_____

Case form

1) Study Number:_____ Hospital number:_____

2) Doctor completing form:_____ 3) Date:_____

4) Informant: Patient / real parent / adoptive parent / carer (but not parent) / spouse or cohabitee / other relative / other (give details:_____)

All the questions below are about the patient, not about the individual providing the answers (unless that is the patient)

5) Surname:_____ 6) Middle Name:_____ 7) First Name:_____

8) Age:____yrs ____months 9) Sex: M / F

10) Ethnic group:_____ 9b) Pregnant?: Y/N 9c) Months pregnant:____

11) Head of Household:_____ 12) Nearest local landmark:_____

14) Group/House Number:_____ _____

15) Hamlet/Street name:_____ _____

16) Commune/Ward:_____ 13) Detailed directions to house:_____

17) Provincial town:_____ _____

18) District:_____ _____

19) City/Province:_____ _____

Follow up team informed yet? ☐

20) Name of biological mother:_____ 21) Maternal ethnic group:_____

22) Name of biological father:_____ 23) Paternal ethnic group:_____

24) Migrant? C/K 24b) From where?:_____ 24c) When (Year)?_____

24d) Why? Government resettled / Marriage / Spontaneous economic / Spontaneous other

25) Occupation:_____

26) Do you stay away from your home for work? Y/N

26b) How much time do you spend away from home? __nights/week __weeks/month __months/year

Notes:_____

Address when away from home:

26c) Name of head of household:_____ 26d) Name of Head of Group:_____

26e) Group/House Number:_____ 26h) Provincial town:_____

26f) Hamlet/Street Name:_____ 26i) District:_____

26g) Commune/Ward:_____ 26j) City/Province:_____

26k) Bed net use in working environment? Y/N 26l) Appropriate / Inappropriate

27) Previous Malaria? C/K 27b) When?:_____

27c) Malaria diagnosed on: Clinical / blood test / don't know

28) Bed net use? C/K 28b) Appropriate / Inappropriate

29) Any treatment in last 2 weeks?: Y/N

29b) What for?: Malaria / RTI / Diarrhoea / Other fever / Injury / Chronic illness

Where did you receive your treatment (multiple answers possible)? 29c) Hospital Y/N

29d) Health Station Y/N 29di) Which one?_____ 29g) Traditional herbal treatment Y/ N

29e) Bought medicine from the market/pharmacyY/N 29f) Bought medicine from a private HCW Y/N

30) Date and time of admission: ___:___ ___/___/___ 31) Admitted from:_____

32) Height: _____ Date measured: _____ 33) Weight:_____ Date measured:_____

Clinical information

Complete all sections for which the patient meets the admission criteria.

Cerebral Malaria ☐

Hours since admission	6	12	18	24	36	48
Glasgow coma score or paediatric coma score	____/15 ____/5	____/15 ____/5	____/15 ____/5	____/15 ____/5	____/15 ____/5	____/15 ____/5

Date and time of LP: ___/___/___ ___:___ Opening pressure:____cm CSF

CSF WBC:____/µl N:___% L:___% CSF glucose/plasma glucose:___/___

Seizures? Y / N

Day	Day before admission	Day of admission	Day after admission	Day 3 of admission
Number of seizures				
Number of seizures witnessed by health care workers				

Renal Failure ☐

Hours since admission	6	12	18	24	36	48
Urine output (mls)						

Highest creatinine:_____

Severe Anaemia ☐ Lowest Hb:_____ OR Lowest Hct:_____

Blood transfusion? Y / N Number of Units:____

EDTA sample for haemoglobinopathy analysis taken before transfusion? Y/N

Macroscopic haemoglobinuria ☐ G6PD result: Normal / Abnormal

Shock ☐ Details:

Time since admission:						
Blood pressure (mmHg)	/	/	/	/	/	/

Jaundice ☐ Bilirubin:_____

Hypoglycaemia ☐

Time since admission						
Plasma glucose						

Respiratory distress ☐

Hours since admission						
Resp rate						
Resp distress?	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N

CXR result:_____

Spontaneous bleeding Bleeding sites:_____

Hyperlactataemia Highest Lactate:_____

Smear taken at: Health station / Hospital

Smear taken: Before treatment / after commencing treatment

Falciparum initial smear result only

Trophozoites:____ Schizonts:____ Gametocytes:____

Parasite Density:_____/400 WBC's _____/1000 RBC's

Blood culture taken? Y/N Blood culture result: Positive / Negative / Contaminant

Blood culture result details:_____

EDTA sample for haemoglobinopathy analysis: Y / N

KAP study forms

List of family members

The interviewer should question the respondent about each family member in turn

	Name	Age(d/m/y)	Ethnic group (1)	Sex	Pregnant?	Relationship to head of house	Immigrant ? (2)	Occupation (3)	Sleep under bednet at home? (4)	Sleep in fields? (5)	Sleep in forest? (5)	Sleep under bednet in field or forest? (4)	Ever had malaria ? (6)	Had malaria this year? (7)	Been to health station this year? (7)	Notes
1					Y/N											
2					Y/N											
3					Y/N											
4					Y/N											
5					Y/N											
6					Y/N											
7					Y/N											
8					Y/N											
9					Y/N											
10					Y/N											
11					Y/N											
12					Y/N											
13					Y/N											
14					Y/N											

(1) K-Kinh, S-S'Tiêng, T-Tày, N-Nùng, Kh-Khmer/Campuchea, M-M'Nông, O-Other
(2) If in-migrant, please document year moved to the area.
(3) Occupation: Agricultural labourer; Smallholder; Wood cutter; Trader; Student; Child; Other
(4) Please note regularity of bednet use: Always, Usually, Sometimes or Never.
(5) If sleeping in field or forest, please indicate frequency, for instance "2/ week" for twice a week, or "2/ month" for twice a month.
(6) If so, please indicate date of last malaria episode.
(7) If so, please indicate the number of times the individual suffered malaria in the last year

Household Questionnaire

Date: ---/---/---

Group _____ Form completed by:_____

Hamlet _____ Minutes walk to nearest main road _____

Commune _____ Head of household _____

Respondent _____ Number of individuals in household _____

1/ Family income

1.1 Does the family own farm land? YesÙ ☐ No☐

If Yes, approximately how much land_____ (1ha, 1maãu = 10.000 m^2)

Does the household grow: Cashew☐ Pepper☐ Rice ☐ cassava☐ Other _____

Approximately how much money, in total does the household earn per year?_____

1.2 How many of the following does the family own? Bicycle☐; Motorbike☐; TV☐; Radio ☐;

Other _____

1.3 Does the family grow enough rice for its needs? Yes☐ No☐

If not, how much rice must the household buy each year?_____

1.4 How often does the family buy rice? Daily ☐; Every 2 days ☐; Weekly ☐; Other _____

How much do you buy at one time? _____

1.5 Does the family have enough food to eat all year round? Yes ☐ No☐

If not, during which months do you not have enough food? _____

2/ Bed net use

2.1 Does the family sleep under bednets? Yes ☐ No ☐

2.2 How many nets does the family own? _____ *Interviewer count:*_____

2.3 When were the nets last treated? Never ☐;<6 months ag0 ☐; 6-12 months ago ☐; > 1year ago☐

What other chemicals does the family use? Mosquito coils☐;Insecticides☐; Pesticides ☐;

Other _____

Details of interviewers inspection of bednets- note number in each condition

Pristine☐; Good ☐; Bad ☐; Terrible ☐ Can't tell ☐

Notes on bednet condition _____ _____ _____

3/ Retiring times

3.1 When do the children in the household usually go to bed? < 8pm ☐; 8pm-12am ☐; >12am☐; Don't know ☐ What time did the children go to bed last night? _____

3.2 When do the adults in the household usually go to bed? <9pm ☐; 9pm-12am ☐; >12am☐; Don't know ☐ What time did the adults go to bed last night? _____

4/ Health care seeking behaviour (for any illness, not just malaria)

4.1 If you are ill, where do you go? Health station ☐; Hospital ☐; Private office[1] ☐; Traditional healer ☐; Depends on illness ☐; Other / notes _____

4.2 If your child was ill, were would you take them? Health station ☐; Hospital ☐; Private office ☐; Traditional healer ☐; Depends on illness ☐; Other / notes _____

4.3 When was the last time an adult in the household had to take medicine? _____

4.4 Where was that medicine bought?: Market ☐; Private doctor ☐; Bought at health station ☐; provided free at health station ☐ Reason for taking medicine? _____

4.5 When was the last time a child in the household had to take medicine? _____

Where was that medicine bought?: Market ☐; Private doctor ☐; Bought at health station ☐; provided free at health station ☐ Reason for taking medicine? _____

4.6 When was the last time anyone in the house had to go to the health station?_____

Why did they have to go? _____

5/ House construction

5.1 How many rooms does your house have? _____

5.2 Do you raise: Cattle/buffalo ☐; Pigs ☐; Other_____

5.3 How far is the animal pen from the house (metres) _____ No animal pen ☐

6/ Observations of the interviewer on house construction

6.1 Area of house : <30 m^2 ☐; 30 – 50 m^2 ☐; >50m^2 ☐;

6.2 Roof constructed of : Sheet metal ☐; Thatch ☐; Woodã ☐; Tile ☐; Other ☐; Notes _____

*6.3 Walls constructed of : Bamboo ☐; Wattle ☐; Wood ☐; Brick ☐; Other ☐; Notes*_____

*6.4 Windows (multiple choices): No windows ☐; Open windows ☐; Bamboo shutters ☐; Wooden shutters ☐; Glass windows ☐; Other ☐;Notes*_____

6.5 Openness score (cracks in walls, wall construction, windows, eaves) - score from 1-very closed to 5-very open _____

6.6 Distance from house to nearest:

Stream _____mt ; Field _____mt; Type of field ? _____; Other(describe _____)____m

[1] This is a direct translation from the Vietnamese, and can mean a private doctor, but also any health care worker who has set themselves up to provide a treatment service.

Individual Questionnaire

Head of household _____ **Interviewer** _____

Commune_____ Date ---/---/---

Hamlet _____ Age: ☐☐/☐☐/☐☐☐☐

Name _____ Sex: Male ☐; Female ☐

1/ Personal information

1.1 Is this your house? Yes ☐ No ☐

If not: Where is your house? _____

How long have you been here? _____

1.2 Where you born and brought up in the area? Yes ☐ No ☐

If not: How long have you lived here? _____

Where are you from? *(hamlet, commune, district, province)* _____

1.3 What level of schooling have you had? None☐; Primary ☐; Middle ☐; High school graduate ☐; University☐

Can you read and write Vietnamese? Yes ☐ No ☐

1.4 Occupation: Agricultural labourer ☐; Smallholder ☐; Wood cutter ☐; Trader ☐ ; Student ☐ Other _____

2/ Transport and field/forest activity

2.1 How do you get around?: On foot ☐; By bicycle ☐; By motorbike ☐; Other _____

2.2 Do you work in the fields ? Yes ☐ No ☐ (If no, proceed to question 2.3)

How long does it take you to reach your fields (minutes)? _____

How often do you go? Daily ☐ 2/week ☐ Weekly ☐ Monthly ☐ Depends on season ☐ Other_____ Notes_____

How often do you sleep in the fields? Often ☐ Rarely☐ Depends on season ☐

Do you use a bednet when you sleep in the field? Yes ☐ No ☐

Where do you sleep when you are at the fields? In a house ☐; In a plot hut ☐; In the open ☐; Other ☐ Notes_____

What do you do if you fall in when you are in the field? Come home and treat myself ☐; Treat myself in the field ☐; Go to the health station ☐; Other (give details)_____

2.3 Do you work in the forest ? Yes ☐ No ☐ (If no, proceed to question 3)

How long does it take you to reach the forest (minutes)? _____

How often do you go? Daily ☐ 2/week ☐ Weekly ☐ Monthly ☐ Depends on season ☐ Other_____ Notes_____

How often do you sleep in the forest? Often ☐ Rarely☐ Depends on season ☐

Do you use a bednet when you sleep in the forest? Yes ☐ No ☐

Where do you sleep when you are at the forest? In a house ☐; In a plot hut ☐; In the open ☐; Other ☐ Notes_____

What do you do if you fall in when you are in the forest? Come home and treat myself ☐; Treat myself in the field ☐; Go to the health station ☐; Other (give details)_____

Individual questionnaire: page 2

3/ Sleeping habits

3.1 What time do you usually go to bed? <9pm☐; 9pm-12am☐; > 12am☐; don't remember☐

What time did you go to bed last night? _____

3.2 Do you use a bednet ? Yes ☐; No ☐ (If not, continue to question 3.3)

If so, is your bednet treated? ☐; Bednet notes _____

3.3 Where did you get your bednet? _____

3.4 Do you ever sleep away from your home? Yes ☐; No ☐ (If not, continue to question 4.1)

Where do you sleep? Another house ☐; In the field ☐; In the forest ☐; Somewhere else ☐
Notes _____

How often do you sleep there?:_____

For how long will you sleep there?: _____

Where do you sleep when you are away?: In a house ☐; In a plot hut ☐; In the open ☐; Other ☐
Notes_____

Do you use a bednet there? Yes ☐; No ☐

4/ Malaria experience

4.1 Have you ever had malaria? Yes ☐ No ☐

If yes, how many times? Once ☐; Twice ☐; 3-5 times ☐; >5 ☐; can't remember ☐

4.2 When was the last time you had malaria? _____

4.3 Who diagnosed the malaria? I did ☐; Health care worker ☐; Other _____

4.4 Where were you treated? _____

4.5 Did you have to pay for your treatment? Yes ☐; No ☐(treated for free)

If "Yes", did you have to pay for the antimalarial medicines? Yes☐; No ☐ Don't know ☐

4.6 How long did you take medicine for? 1-2 days☐; 3-6 days☐; More than 7 days ☐

4.7 When was the last time you went to the health station? _____ _____

What did you go for? _____ _____ _____

5/ Malaria knowledge

5.1 Do you know what causes malaria? Yes ☐; No ☐

If so, please give details _____

5.2 Do you think malaria is preventable? Yes ☐; No ☐

5.3 Do you think malaria is treated for free at health stations and hospitals? Yes ☐; No ☐

Appendix 2

Additional tables

Concept	Per survey statistic	Combine survey statistic	Advantages	Disadvantages
Smear result vs expected smear result	p_s-r_s	Average	Conceptually simple	Not normal No theoretical basis for averaging
Smear result vs expected smear result	p_s-r_s	Product		Per survey stat not a probability Negative per survey stat leads to sign changes Far from clear how this is distributed
Actual number of positive smears vs expected number of positive smears	N/A	$\sum_s r_s - \sum_s p_s$	Intuitively reasonable way to combine survey experiences	Unclear that summing p_s represents expected number of smear positive episodes Most subjects will have been smear negative at all visits, thus most values will be negative Unclear how statistic will be distributed
Raw probabilities of event	p_s if smear pos 1- p_s if negative	Product	Mathematically pure	Summary statistic intuitively wrong: subject smear negative in 3 surveys could have summary statistic of 0.5 (lower values reflect greater chance of smear positivity), whereas subject appearing in only one, later survey could have statistic of 0.94, despite possibly having been smear positive in the past during times of greater prevalence
Probabalistically calculated innate "smearness"	N/A	$\prod_s (1-p_s)$ if never smear positive $1-\prod_s (1-p_s)$ if ever smear positive	Mathematically pure Clearly separates smear positive and negative individuals without introducing bias	Discards information from multiple positive smears Similar problem to above with regard to repeatedly negative individuals
Estimate innate "smearness" through maximum likelihood methods			Uses data from multiple smear positive events Robust estimate	Requires multiple observations, and most individuals only appear in one survey Discards data from multiply negative events (probably not a severe problem)

Table 1a. Methods of combining smear data from previous surveys for individual subjects in KAP study (p_s=probability of being smear positive in survey s, r_s=result (0 or 1) of blood smear in survey s).

Concept	Per survey statistic	Combine survey statistic	Advantages	Disadvantages (all suffer from what value p_s should take – see text)
Smear result vs expected smear result	$p_s - \frac{\sum_{i=1}^{i=n_{hs}} r_{si}}{n_{hs}}$	Average	Conceptually simple Intuitively reasonable way to compare household experiences with individual survey risk	Not normal No theoretical basis for averaging Many households will have few representatives – if only one will reduce to individual probability, if more than one will be discontinuous in quanta of $1/n_{hs}$
Smear result vs expected smear result	$p_s - \frac{\sum_{i=1}^{i=n_{hs}} r_{si}}{n_{hs}}$	Product		Same issues as equivalent statistic for individuals
Actual proportion of positive smears vs expected proportion of positive smears	N/A	$\frac{\sum_{si} r_{si}}{\sum_{s} n_{hs}} - \sum_{s} p_s$	Intuitively reasonable way to combine surveys	Most subjects will have been smear negative at all visits, thus most values will be negative Unclear how statistic will be distributed
Raw probabilities of event	$\frac{np_{hs}!}{np_{hs}!.nn_{hs}!}.p_s^{np_{hs}}$ x $\frac{nn_{hs}!}{np_{hs}!.nn_{hs}!}.(1-p_s)^{nn_{hs}}$	Product	Mathematically pure	Summary statistic intuitively wrong for same reasons as use of raw probabilities in individuals
Estimate innate "household smearness" through maximum likelihood methods			Uses multiple positive events. Robust estimate	Requires multiple observations, and most households only appear in one survey, and only have one representative per survey (but higher proportion of households ever positive than individuals). Discards data from persistently negative households (probably not a severe problem)

Table 1b. Methods of combining previous survey smear data at household level for households in KAP study (p_s as above, r_{si}=result of individual i in survey s, n_{hs}=number of subjects in household h in survey s, np_{hs}=number of subjects with positive blood smears in household h in survey s, nn_{hs}=number of subjects with negative blood smears in household h in survey s.

Village	Hamlet	Total	Kinh	S'Tiêng	Tày	Nùng	Khmer	M'Nông
Đồng Tâm	1	113	52 (46.0%, 3.2%)	13 (11.5%, 1.2%)	22 (19.5%, 5.2%)	22 (19.5%, 8.2%)	1 (0.9%, 1.8%)	1 (0.9%, 0.2%)
	2	185	9 (4.9%, 0.6%)	79 (42.7%, 7.0%)	17 (9.2%, 4.0%)	80 (43.2%, 29.7%)	0	0
	3	109	103 (94.5%, 6.3%)	0	5 (4.6%, 1.2%)	0	1 (0.9%, 1.8%)	0
	4	257	143 (55.6%, 8.7%)	75 (29.2%, 6.6%)	10 (3.9%, 2.4%)	5 (1.9%, 1.9%)	22 (8.6%, 38.6%)	0
	5	88	46 (52.3%, 2.8%)	42 (47.7%, 3.7%)	0	0	0	0
	6	155	87 (56.1%, 5.3%)	45 (29.0%, 4.0%)	0	0	23 (14.8%, 40.4%)	0
	Cầu 2	101	73 (72.3%, 4.5%)	0	25 (24.8%, 6.0%)	2 (2.0%, 0.7%)	0	0
	Suối Binh	127	0	0	69 (54.3%, 16.4%)	58 (45.7%, 21.6%)	0	0
	Suối Đôi	138	8 (5.8%, 0.5%)	0	117 (84.8%, 27.9%)	13 (9.4%, 4.8%)	0	0
Đak Nhâu	Bù Ghe	61	24 (39.3%, 1.5%)	2 (3.3%, 0.2%)	7 (11.5%, 1.7%)	0	0	26 (42.6%, 4.5%)
	Bù Oai	240	124 (51.7%, 7.6%)	64 (26.7%, 5.7%)	24 (10.0%, 5.7%)	6 (2.5%, 2.2%)	0	0
	Dak La	122	23 (18.9%, 1.4%)	0	3 (2.5%, 0.7%)	1 (0.8%, 0.4%)	0	91 (74.6%, 15.8%)
	Dak Ma	108	94 (87.0%, 5.7%)	0	9 (8.3%, 2.1%)	0	0	0
	Dak Nùng	92	22 (23.9%, 1.3%)	0	0	0	0	68 (73.9%, 11.8%)
	Dak Wí	89	29 (32.6%, 1.8%)	11 (12.4%, 1.0%)	35 (39.3%, 8.3%)	9 (10.1%, 3.3%)	0	0
	Dak Xuyên	132	44 (33.3%, 2.7%)	11 (8.3%, 1.0%)	3 (2.3%, 0.7%)	1 (0.8%, 0.4%)	0	66 (50.0%, 11.5%)
	Dang Lang	183	9 (4.9%, 0.6%)	3 (1.6%, 0.3%)	0	0	0	171 (93.4%, 29.7%)
	Đắc Liên	175	23 (13.1%, 1.4%)	0	0	0	0	152 (86.9%, 26.4%)
	Thống Nhất	76	41 (53.9%, 2.5%)	0	6 (7.9%, 1.4%)	26 (34.2%, 9.7%)	1 (1.3%, 1.8%)	0

Table 2a. Ethnic group breakdown by hamlet of the first survey sample (majority Kinh and more numerous ethnic minorities). Cell contents are number of subjects (percentage of hamlet sample, percentage of ethnic group sample in the specified hamlet). Đồng Tâm and Đak Nhau only – see below for Đắc Ơ

Village	Hamlet	Total	Kinh	S'Tiêng	Tày	Nùng	Khmer	M'Nông
Đắc Ơ	3	123	17 (13.8%, 1.0%)	78 (63.4%, 6.9%)	22 (17.9%, 5.2%)	5 (4.1%, 1.9%)	0	0
	4	286	18 (6.3%, 1.1%)	188 (65.7%, 16.7%)	30 (10.5%, 7.1%)	37 (12.9%, 13.8%)	0	0
	6	108	84 (77.8%, 5.1%)	17 (15.7%, 1.5%)	4 (3.7%, 1.0%)	0	0	0
	7	31	20 (64.5%, 1.2%)	5 (16.1%, 0.4%)	6 (19.4%, 1.4%)	0	0	0
	9	152	136 (89.5%, 8.3%)	10 (6.6%, 0.9%)	1 (0.7%, 0.2%)	0	5 (3.3%, 8.8%)	0
	Bù Bưng	219	28 (12.8%, 1.7%)	191 (87.2%, 16.9%)	0	0	0	0
	Bù Ka	191	5 (2.6%, 0.3%)	186 (97.4%, 16.5%)	0	0	0	0
	Bù Khơn	116	11 (9.5%, 0.7%)	100 (86.2%, 8.9%)	0	0	4 (3.4%, 7.0%)	0
	Bù Xia	129	128 (99.2%, 7.8%)	0	0	0	0	0
	Đắc Ku	185	174 (94.1%, 10.6%)	8 (4.3%, 0.7%)	1 (0.5%, 0.2%)	0	0	0
	Đắc Lim	69	61 (88.4%, 3.7%)	0	4 (5.8%, 1.0%)	4 (5.8%, 1.5%)	0	0

Table 2b. Ethnic group breakdown by hamlet of the first survey sample (majority Kinh and more numerous ethnic minorities). Cell contents are number of subjects (percentage of hamlet sample, percentage of ethnic group sample in the specified hamlet)

Village	Hamlet	Total	Chăm	Dao	Hoa	Mường	Other (total in sample 5 or fewer)
Đồng Tâm	1	113	0	0	0	0	1 Châu-ro
	2	185	0	0	0	0	
	3	109	0	0	0	0	
	4	257	1 (0.4%, 9.1%)	0	0	1 (0.4%, 10.0%)	
	5	88	0	0	0	0	
	6	155	0	0	0	0	
	Cầu 2	101	0	0	0	1 (1.0%, 10.0%)	
	Suối Binh	127	0	0	0	0	
	Suối Đôi	138	0	0	0	0	

Table 2c. Ethnic group breakdown by hamlet of the first survey sample (less numerous ethnic minorities). Cell contents are number of subjects (percentage of hamlet sample, percentage of ethnic group sample in the specified hamlet). Đồng Tâm only – see below for Đak Nhau and Đắc Ơ.

Village	Hamlet	Total	Chăm	Dao	Hoa	Mường	Other (total in sample 5 or fewer)
Đak Nhâu	Bù Ghe	61	0	0	2 (3.3%, 12.5%)	0	
	Bù Oai	240	0	19 (7.9%, 90.5%)	2 (0.8%, 12.5%)	1 (0.4%, 10.0%)	
	Dak La	122	0	0	4 (3.3%, 25.0%)	0	
	Dak Ma	108	0	1 (0.9%, 4.8%)	0	0	4 Sáu Dìu
	Dak Nùng	92	0	0	1 (1.1%, 6.3%)	0	1 Thái
	Dak Wí	89	0	0	4 (4.5%, 25.0%)	0	1 Sáu Dìu
	Dak Xuyên	132	0	0	3 (2.3%, 18.8%)	1 (0.8%, 10.0%)	3 Ê Đê
	Dang Lang	183	0	0	0	0	
	Đắc Liên	175	0	0	0	0	
	Thống Nhất	76	0	1 (1.3%, 4.8%)	0	0	1 Thương
Đak Ơ	3	123	1 (0.8%, 9.1%)	0	0	0	
	4	286	9 (3.1%, 81.8%)	0	0	4 (1.4%, 40.0%)	
	6	108	0	0	0	0	2 Chao Mà 1 Tìu
	7	31	0	0	0	0	
	9	152	0	0	0	0	
	Bù Bưng	219	0	0	0	0	
	Bù Ka	191	0	0	0	0	
	Bù Khơn	116	0	0	0	0	1 Chao Mà
	Bù Xia	129	0	0	0	1 (0.8%, 10.0%)	
	Đắc Ku	185	0	0	0	1 (0.5%, 10.0%)	
	Đắc Lim	69	0	0	0	0	

Table 2d. Ethnic group breakdown by hamlet of the first survey sample (less numerous ethnic minorities). Cell contents are number of subjects (percentage of hamlet sample, percentage of ethnic group sample in the specified hamlet)

Hamlet	Falciparum alone	Vivax alone	Mixed Pf/Pv	Total
Đak Xuyền	6	4	0	10
Đak La	8	5	0	13
Bù Ghe	0	3	0	3
Đắc Liên	11	8	3	22
Đak Nùng	9	5	0	14
Đang Lang	8	0	0	8
Total	42	25	3	70

Table 3. Hamlet distribution of smear positive M'Nông in Đak Nhau

Ethnic group	Mean age	SE	p value
Kinh	20.6	1.26	0.007
S'tiêng	14.7	0.93	0.003
Tày	28.4	5.16	0.006
Nùng	26.8	3.63	0.014
M'Nông	14.2	1.65	0.116
Total	17.0	0.67	

Table 4. Mean ages and standard errors of smear positive subjects by ethnic group with p value from T test of mean age of ethnic group vs all other ethnic groups.

Age group	Kinh	S'tiêng	Tày	Nùng	M'Nông	Other	Total
<1	0	4 (1.3%)	0	0	2 (3%)	0	6 (1.0%)
1	2 (1.2%)	10 (3.3%)	0	0	0	1 (9%)	13 (2.3%)
2-4	15 (9.2%)	63 (21.1%)	0	0	13 (18%)	0	91 (15.8%)
5-9	34 (20.9%)	91 (30.4%)	2 (13%)	1 (6%)	25 (35%)	3 (27%)	156 (27.1%)
10-14	27 (16.6%)	34 (11.4%)	4 (27%)	1 (6%)	7 (10%)	2 (18%)	75 (13.0%)
15-19	14 (8.6%)	22 (7.4%)	0	6 (38%)	3 (4%)	1 (9%)	46 (8.0%)
20-29	26 (16.0%)	28 (9.4%)	3 (20%)	1 (6%)	10 (14%)	3 (27%)	71 (12.4%)
30-39	21 (12.9%)	18 (6.0%)	2 (13%)	3 (19%)	6 (9%)	1 (9%)	51 (8.9%)
40-49	13 (8.0%)	8 (2.7%)	1 (7%)	2 (13%)	3 (4 %)	0	27 (4.7%)
50-59	8 (4.9%)	11 (3.7%)	2 (13%)	2 (13%)	1 (1%)	0	24 (4.2%)
60-69	1 (0.6%)	6 (2.0%)	0	0	0	0	7 (1.2%)
70+	2 (1.2%)	4 (1.3%)	1 (7%)	0	1 (1%)	0	8 (1.4%)
Total	163	299	15	16	71	11	575

Table 5. Age and ethnic group breakdown of smear positive subjects. Percentages down the column given.

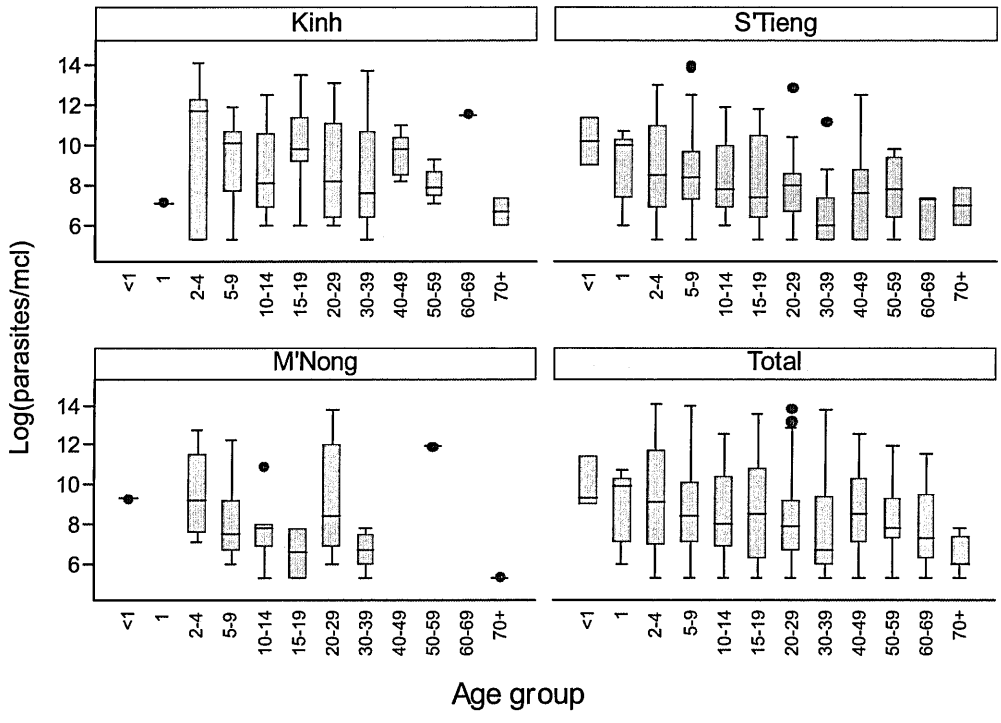

Fig 1. Comparison of age related differences in parasite count between ethnic groups.

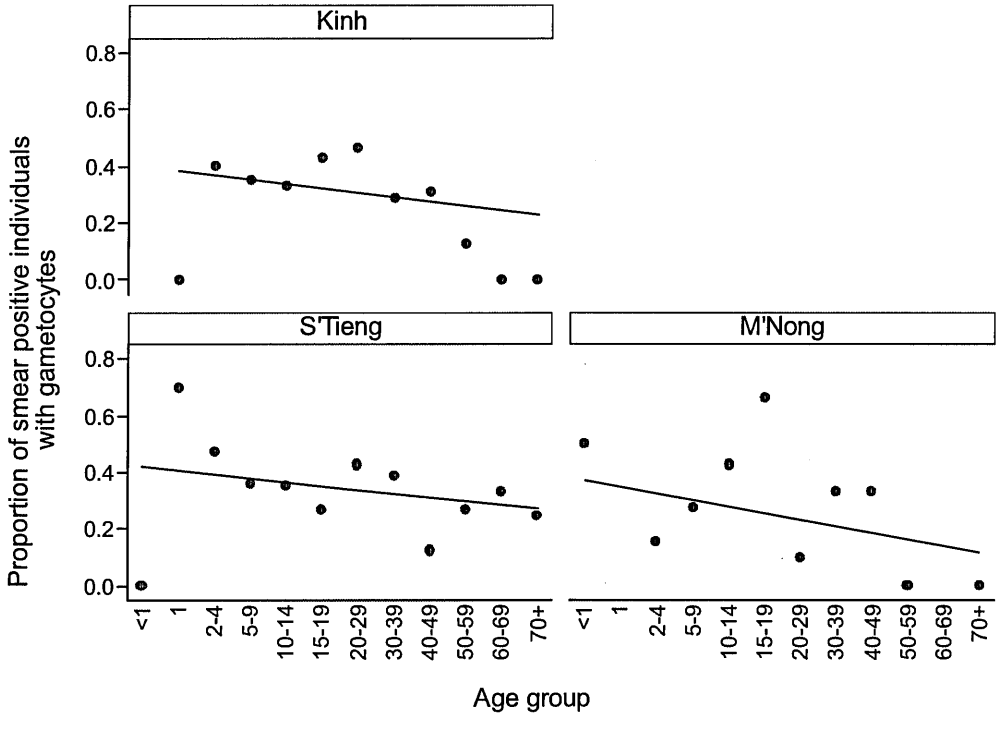

Fig 2. Comparison of age related differences in proportion of smear positive individuals carrying gametocytes between the different ethnic groups.

	Diarrhoea	>5 stools	Sore throat	Earache or discharging ears	Runny nose	Jaundice	Pallor	Inflamed ears	Inflamed throat	Abnormal chest ausculatation	Rigors
<1	22% (18)	0% (18)	15% (13)	0% (14)	86% (21)	0% (21)	14% (21)	0% (21)	5% (21)	25% (16)	7% (15)
1	32% (31)	13% (31)	20% (25)	4% (23)	93% (30)	3% (33)	9% (33)	1% (100)	9% (100)	3% (29)	8% (26)
2-4	20% (83)	4% (70)	27% (79)	4% (71)	82% (83)	2% (83)	7% (83)	3% (32)	28% (32)	9% (74)	27% (82)
5-9	12% (97)	1% (90)	31% (94)	7% (83)	65% (95)	5% (96)	21% (97)	2% (100)	9% (100)	2% (82)	38% (97)
10-14	8% (52)	4% (50)	29% (51)	2% (50)	37% (52)	12% (52)	28% (53)	2% (83)	11% (84)	2% (43)	53% (51)
15-19	11% (36)	0% (32)	23% (35)	0% (27)	29% (34)	14% (36)	22% (36)	4% (100)	19% (100)	0% (27)	65% (34)
20-29	15% (52)	7% (45)	32% (50)	0% (43)	22% (49)	4% (51)	12% (51)	4% (99)	20% (95)	3% (38)	56% (52)
30-39	10% (49)	2% (52)	25% (51)	0% (42)	33% (48)	17% (48)	19% (48)	0% (100)	8% (100)	0% (36)	61% (49)
40-49	10% (30)	0% (29)	30% (30)	4% (24)	17% (29)	24% (29)	24% (29)	0% (55)	15% (55)	0% (22)	52% (29)
50-59	11% (18)	0% (19)	39% (18)	0% (16)	47% (19)	17% (18)	22% (18)	0% (100)	8% (100)	0% (14)	72% (18)
60-69	0% (6)	0% (6)	17% (6)	0% (5)	17% (6)	50% (6)	50% (6)	0% (36)	22% (36)	33% (6)	33% (6)
70+	0% (6)	0% (6)	33% (6)	0% (6)	50% (4)	50% (6)	67% (6)	3% (100)	15% (100)	0% (2)	50% (6)
Total	14% (478)	3% (448)	28% (458)	3% (404)	53% (470)	9% (479)	18% (481)	6% (51)	28% (53)	5% (389)	44% (465)

	Chills	Headache	Pyrexial	Splenomegaly	Reported fever
<1	7% (14)	9% (11)	8% (167)	4% (143)	25% (163)
1	9% (23)	11% (19)	10% (189)	6% (171)	28% (184)
2-4	32% (78)	35% (66)	11% (570)	14% (506)	26% (552)
5-9	44% (84)	55% (91)	8% (859)	16% (778)	18% (840)
10-14	57% (51)	91% (55)	10% (429)	19% (391)	20% (420)
15-19	67% (33)	89% (35)	11% (199)	15% (181)	23% (191)
20-29	72% (47)	91% (53)	8% (534)	6% (483)	18% (524)
30-39	79% (47)	92% (48)	9% (438)	8% (385)	22% (431)
40-49	68% (28)	96% (26)	5% (310)	3% (263)	20% (297)
50-59	75% (16)	94% (18)	4% (225)	6% (203)	15% (220)
60-69	17% (6)	83% (6)	2% (162)	3% (148)	8% (155)
70+	80% (5)	100% (6)	2% (104)	3% (91)	15% (100)
Total	52% (432)	70% (434)	8% (4186)	11% (3743)	20% (4077)

Table 6. Breakdown by age group of all symptoms and signs.

	Diarrhoea	>5 stools per day	Sore throat	Earache or discharging ears	Coryza	Jaundice	Pallor	Inflamed ears	Inflamed throat	Abnormal chest auscultation	Rigors
Kinh	8% (234)	3% (231)	23% (229)	2% (204)	41% (229)	11% (227)	22% (228)	2% (230)	17% (228)	2% (183)	46% (229)
S'tiếng	16% (98)	3% (80)	24% (90)	5% (83)	72% (100)	6% (103)	17% (103)	3% (100)	11% (100)	10% (87)	33% (92)
Tày	22% (51)	2% (47)	29% (48)	2% (42)	51% (49)	2% (50)	4% (50)	3% (105)	10% (105)	2% (43)	35% (51)
Nùng	24% (29)	8% (26)	32% (28)	0% (25)	48% (27)	3% (30)	10% (30)	2% (100)	14% (100)	7% (28)	25% (28)
M'Nông	20% (45)	2% (49)	64% (45)	0% (33)	67% (43)	25% (44)	31% (45)	4% (50)	28% (50)	7% (27)	70% (44)
Other	33% (21)	0% (15)	11% (18)	12% (17)	73% (22)	8% (25)	4% (25)	0% (100)	4% (100)	5% (21)	52% (21)
Total	14% (478)	3% (448)	28% (458)	3% (404)	53% (470)	9% (479)	18% (481)	0% (29)	14% (29)	5% (389)	44% (465)

	Chills	Headache	Pyrexial	Splenomegaly	Reported fever
Kinh	55% (218)	69% (212)	9% (1646)	6% (1423)	25% (1595)
S'tiếng	43% (87)	64% (81)	8% (1129)	23% (1026)	15% (1099)
Tày	48% (46)	68% (50)	5% (423)	3% (394)	14% (418)
Nùng	27% (26)	70% (27)	9% (271)	2% (252)	13% (267)
M'Nông	70% (43)	78% (46)	7% (582)	10% (525)	24% (565)
Other	58% (12)	78% (18)	7% (135)	11% (123)	29% (133)
Total	52% (432)	70% (434)	8% (4186)	11% (3743)	20% (4077)

Table 7. Breakdown by ethnic group of all symptoms and signs

	Diarrhoea	>5 stools per day	Sore throat	Earache or discharging ears	Coryza	Jaundice	Pallor	Inflamme d ears	Inflamed throat	Abnormal chest auscultation	Rigors
Đắc Ơ	8% (142)*	2% (113)	18% (133)	2% (128)	51% (141)	5% (131)	20% (132)	2% (129)	9% (131)	3% (116)	43% (134)
Đồng Tâm	19% (240)	3% (237)	18% (227)	4% (216)	48% (238)	7% (251)	16% (251)	5% (100)	33% (100)*	5% (223)	34% (236)
Đak Nhau	14% (96)	4% (98)	64% (97)*	2% (60)	68% (91)*	23% (97)*	22% (98)	2% (260)	13% (258)	4% (50)	69% (95)*
Total	14% (478)	3% (448)	28% (457)	3% (404)	53% (470)	9% (479)	18% (481)	2% (100)	47% (100)	5% (389)	44% (465)

	Chills	Headache	Pyrexial	Splenomegaly	Fever
Đắc Ơ	61% (135)	72% (117)	8% (1622)	15% (1362)*	19% (1577)
Đồng Tâm	37% (207)*	63% (219)[1]	11% (1274)*	9% (1218)	23% (1242)*
Đak Nhau	71% (90)	83% (98)	6% (1288)	6% (1161)	19% (1256)
Total	52% (432)	70% (434)	8% (4184)	11% (3741)	20% (4075)

Table 8. Breakdown by village of all symptoms and signs

	No fever	Fever	Total
Negative	2,874	640	3,514
Row %	81.8	18.2	
Col %	88.2	78.1	86.2
Positive	383	180	563
Row %	68.0	32.0	
Col %	11.8	22.0	13.8
Total	3,257	820	4,077
Row %	79.9	20.1	p<0.001

Smear	Apyrexial	Pyrexial	Total
Negative	3,377	234	3,611
Row %	93.5	6.5	
Col %	87.8	69.2	86.3
Positive	471	104	575
Row %	81.9	18.1	
Col %	12.2	30.8	13.7
Total	3,848	338	4,186
Row %	91.9	8.1	p<0.001

Smear	No fever in past 2 days	Fever in past 2 days	Total
Negative	3,080	416	3,496
Row %	88.1	11.9	
Col %	88.1	74.4	86.2
Positive	417	143	560
Row %	74.5	25.5	
Col %	11.9	25.6	13.8
Total	3,497	559	4,056
Row %	86.2	13.8	p<0.001

Smear	No fever in past 7 days	Fever in past 7 days	Total
Negative	3,071	430	3,501
Row %	87.72	12.3	
Col %	87.19	80.7	86.3
Positive	451	103	554
Row %	81.41	18.6	
Col %	12.81	19.3	13.7
Total	3,522	533	4,055
Row %	86.9	13.1	p<0.001

Smear	No reported or measured fever	Reported or measured fever	Total
Negative	2,855	756	3,611
Row %	79.1	20.9	
Col %	88.6	78.4	86.3
Positive	367	208	575
Row %	63.8	36.2	
Col %	11.4	21.6	13.7
Total	3,222	964	4,186
Row %	77.0	23.0	p<0.001

Tables 9. Association between clinical features and blood smear result (universally collected data).

Smear	Spleen not palpable	Splenomegaly	Total
Negative	3,001	215	3,216
Row %	93.3	6.7	
Col %	89.7	54.2	85.9
Positive	345	182	527
Row %	65.5	34.5	
Col %	10.3	45.8	14.1
Total	3,346	397	3,743
Row %	89.4	10.6	p<0.001

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Smear	Apyrexial	Pyrexial	Total
Negative	275	127	402
Row %	68.4	31.6	
Col %	82.8	60.8	74.3
Positive	57	82	139
Row %	41.0	59.0	
Col %	17.2	39.2	25.7
Total	332	209	541
Row %	61.4	38.6	p<0.001

Smear	No Rigors	Rigors	Total
Negative	214	128	342
Row %	62.6	37.4	
Col %	81.7	63.1	73.6
Positive	48	75	123
Row %	39.0	61.0	
Col %	18.3	37.0	26.5
Total	262	203	465
Row %	56.3	43.7	p<0.001

Smear	Not jaundiced	Jaundiced	Total
Negative	317	34	351
Row %	90.3	9.7	
Col %	73.0	75.6	73.3
Positive	117	11	128
Row %	91.4	8.6	
Col %	27.0	24.4	26.7
Total	434	45	479
Row %	90.6	9.4	p=0.717

Tables 10a. Associations between symptoms and smear result (selectively gathered data)

Smear	Spleen not palpable	Splenomegaly	Total
Negative	297	58	355
Row %	83.7	16.3	
Col %	83.7	47.5	74.4
Positive	58	64	122
Row %	47.5	52.5	
Col %	16.3	52.5	25.6
Total	355	122	477
Row %	74.4	25.6	p<0.001

Smear	No Chills	Chills	Total
Negative	176	144	320
Row %	55.0	45.0	
Col %	84.2	64.6	74.1
Positive	33	79	112
Row %	29.5	70.5	
Col %	15.8	35.4	25.9
Total	209	223	432
Row %	48.4	51.6	p<0.001

Smear	No Pallor	Pallor	Total
Negative	304	47	351
Row %	86.6	13.4	
Col %	77.4	53.4	73.0
Positive	89	41	130
Row %	68.5	31.5	
Col %	22.7	46.6	27.0
Total	393	88	481
Row %	81.7	18.3	p<0.001

Smear	No Diarrhoea	Diarrhoea	Total
Negative	299	54	353
Row %	84.7	15.3	
Col %	73.1	78.3	73.9
Positive	110	15	125
Row %	88.0	12.0	
Col %	26.9	21.7	26.2
Total	409	69	478
Row %	85.6	14.4	p=0.367

Smear	No Headache	Headache	Total
Negative	108	210	318
Row %	34.0	66.0	
Col %	81.8	69.5	73.3
Positive	24	92	116
Row %	20.7	79.3	
Col %	18.2	30.5	26.7
Total	132	302	434
Row %	30.4	69.6	p=0.008

Smear	No coryzal symptoms	Coryza	Total
Negative	150	193	343
Row %	43.7	56.3	
Col %	67.9	77.5	73.0
Positive	71	56	127
Row %	55.9	44.1	
Col %	32.1	22.5	27.0
Total	221	249	470
Row %	47.0	53.0	p=0.019

Smear	<6 stools/day	>5 Stools	Total
Negative	327	10	337
Row %	97.0	2.97	
Col %	75.4	71.4	75.2
Positive	107	4	111
Row %	96.4	3.6	
Col %	24.7	28.6	24.8
Total	434	14	448
Row %	96.9	3.1	p=0.738

Smear	Chest sounds normal	Abnormal chest ausculation	Total
Negative	271	16	287
Row %	94.4	5.6	
Col %	73.1	88.9	73.8
Positive	100	2	102
Row %	98.0	2.0	
Col %	27.0	11.1	26.2
Total	371	18	389
Row %	95.4	4.6	p=0.136

Tables 10b. Associations between symptoms and smear result (selectively gathered data)

Smear	Throat not sore	Sore throat	Total
Negative	239	97	336
Row %	71.1	28.9	
Col %	72.4	75.8	73.4
Positive	91	31	122
Row %	74.6	25.4	
Col %	27.6	24.2	26.6
Total	330	128	458
Row %	72.1	28.0	p=0.466

Smear	No earache/ aural discharge	Earache or aural discharge	Total
Negative	286	9	295
Row %	97.0	3.1	
Col %	73.0	75.0	73.0
Positive	106	3	109
Row %	97.3	2.8	
Col %	27.0	25.0	27.0
Total	392	12	404
Row %	97.0	3.0	p=0.875

Smear	Normal TM	Inflamed TM	Couldn't examine	Total
Negative	278	8	71	357
Row %	77.9	2.2	19.9	
Col %	73.0	80.0	77.2	73.9
Positive	103	2	21	126
Row %	81.8	1.6	16.7	
Col %	27.0	20.0	22.8	26.1
Total	381	10	92	483
Row %	78.9	2.1	19.1	p=0.565

Smear	No pharyngitis	Pharyngitis	Couldn't examine	Total
Negative	261	73	20	354
Row %	73.7	20.6	5.7	
Col %	71.9	79.4	76.9	73.6
Positive	102	19	6	127
Row %	80.3	15.0	4.7	
Col %	28.1	20.7	23.1	26.4
Total	363	92	26	481
Row %	75.5	19.1	5.4	p=0.051

Table 10c. Associations between symptoms and smear result (selectively gathered data)

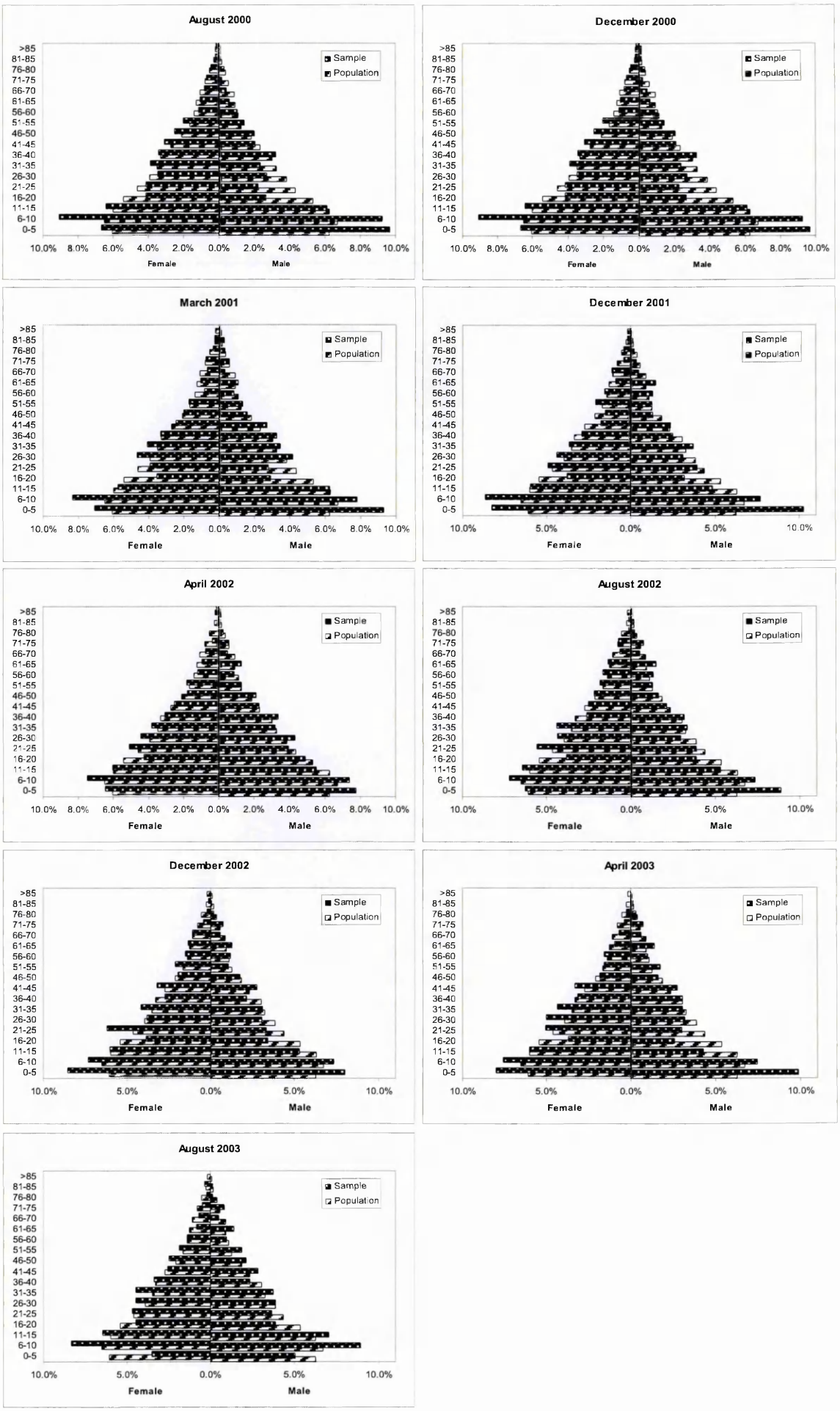
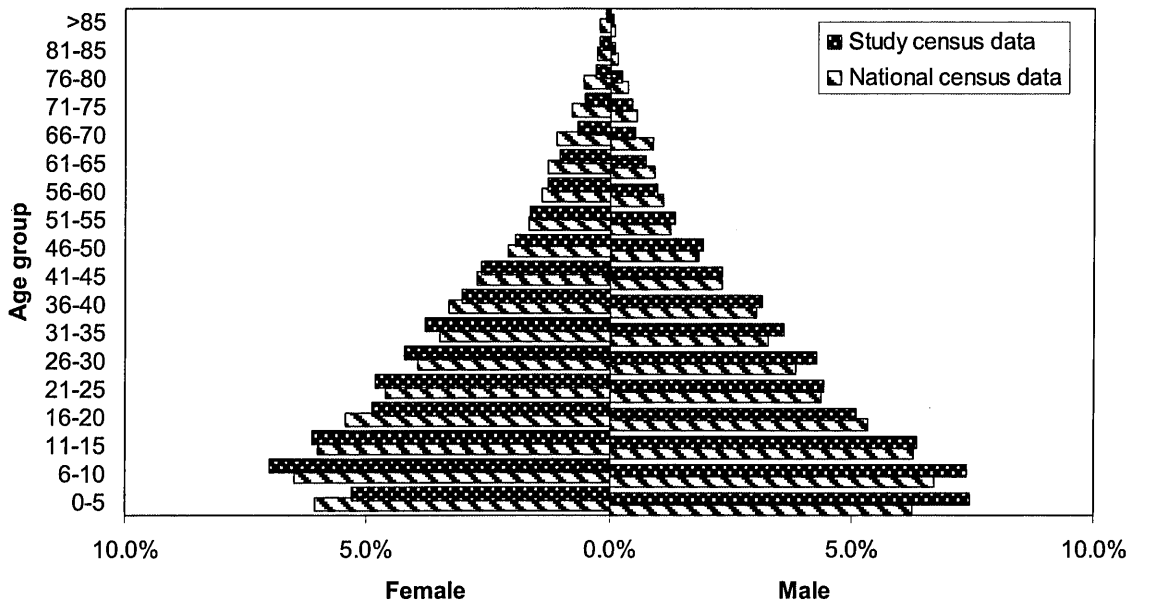
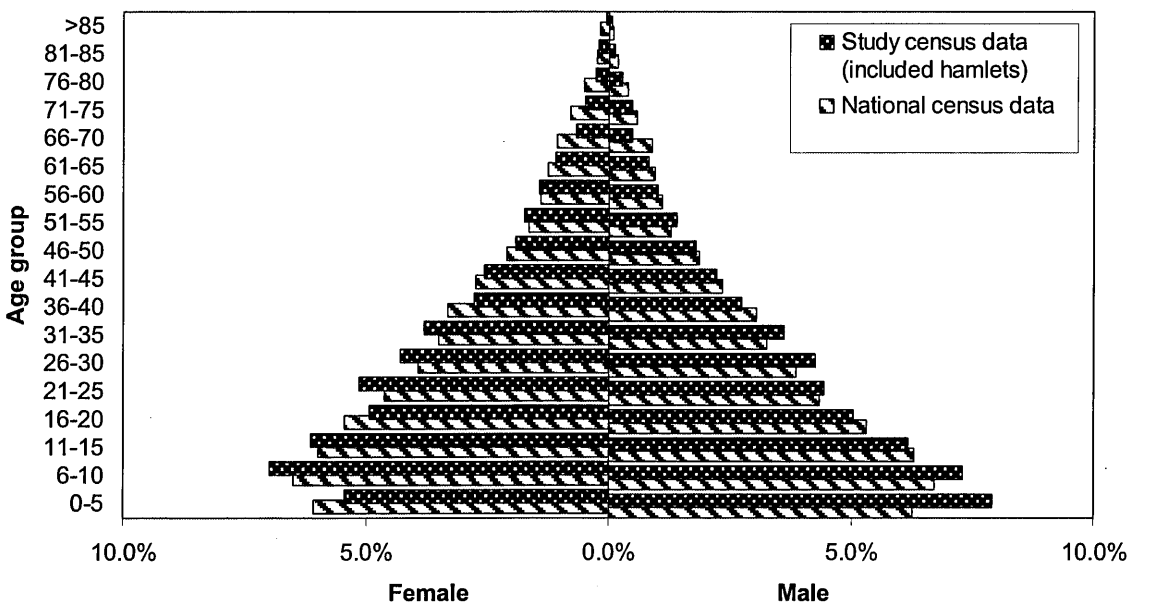


Fig 4. Individual age and sex pyramids for each survey





Figs 5. Study site census data for all hamlets (above) and for only those hamlets included after December 2000 (below) compared to a population pyramid calculated from national age and sex data for the different ethnic groups, weighted according to the ethnic mix of the study population.

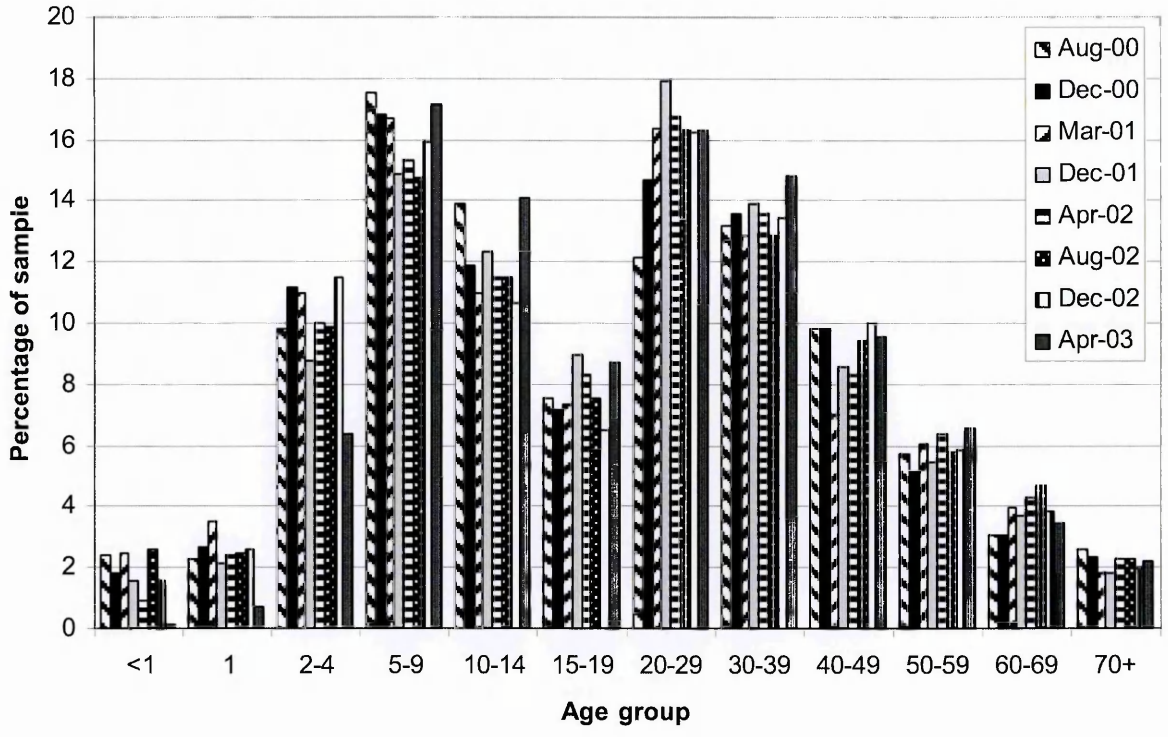


Fig 6. Age distribution of samples in different surveys.

Ethnicity	Symptomatic	Presymptomatic	Asymptomatic	Total
Kinh	38%	24%	38%	29
S'tiêng	38.4%	31.4%	30.2%	172
Tày	20%	0	80%	5
Nùng	62%	8%	31%	13
Other	50%	0	50%	2
Total	39.4%	28.1%	32.6%	221

Table 11. Symptom status by ethnic group (headache alone excluded)

	Species	Symptomatic	Presymptomatic	Asymptomatic	Total
Any symptom	P.falciparum	48.5%	30.0%	21.5%	130
	P.vivax	44%	32%	24%	66
	Mixed	48%	26%	26%	23
	Total	46.9%	30.8%	22.3%	224
Excluding headache alone	P.falciparum	40.6%	25.8%	33.6%	128
	P.vivax	38%	29%	33%	66
	Mixed	39%	30%	30%	23
	Total	39.6%	27.9%	32.4%	222

Table 12. Symptom status by species of parasite on initial smear

Hamlet	Kinh	S'tiếng	Tày	Nùng	Other	Total
Thôn 3	66.7% (3)	40.4% (89)	20.0% (5)			40.2% (97)
Thôn 4	100% (1)	38.9% (18)	20.0% (5)	62.5% (8)	0 (1)	42.4% (33)
Thôn 6	83.3% (6)	40.5% (42)	100% (1)		100% (1)	48.0% (50)
Thôn 7	100% (2)	50.0% (4)	100% (4)	100% (1)		81.8% (11)
Thôn 9	45.5% (22)	33.3% (3)				44.0% (25)
Bù Bưng	100% (1)	44.4% (72)				45.2% (73)
Bù Cà		34.1% (82)				34.1% (82)
Bù Khơn		47.2% (125)				47.2% (125)
Bù Xia	54.5% (11)					54.5% (11)
Đak Lim	100% (1)					100% (1)
Đak U	63.2% (19)					63.2% (19)
Ấp 3 Phú Văn	100% (1)					100% (1)
19 Tháng 5						
Bình Đức 1	100% (2)					100% (2)
Bình Đức 2	0 (3)					0 (3)
Bù Ca Mau		44.7% (38)			100% (1)	46.2% (39)
Bù Gia Phúc	0 (1)	51.4% (74)				50.7% (75)
Bù Gia Phúc 1		60.0% (25)				60.0% (25)
Bù Gia Phúc 2	100% (1)	43.8% (16)				47.1% (17)
Bù Ca Roi		55.7% (88)				55.7% (88)
Đak KhÂu	0 (1)	42.9% (35)				41.7% (36)
Đắc Sơn 1		61.0% (77)				61.0% (77)
Đắc Sơn 2		51.2% (43)				51.2% (43)
Đức Lập	75.0% (8)					75.0% (8)
Khăc Khoan	100% (1)					100% (1)
Thôn 4 Khăc Khoan		33.3% (3)				33.3% (3)
Phước Sơn						
Phú Nghĩa		25.0% (28)			0 (1)	24.1% (29)
Sóc 2 Căn	16.7% (6)	34.4% (32)			0 (2)	30.0% (40)
Sơn Trung		44.4% (27)				44.4% (27)
Thác Dài		100% (3)				100% (3)
Tân Lập		100% (1)				100% (1)
Thôn 2 ĐK	77.8% (9)					77.8% (9)
Thôn 3 ĐK	37.5% (8)	0 (1)			100% (1)	40.0% (10)
Thôn 4 ĐK			0 (1)			0 (1)
Thôn 5 ĐK						
Thôn 6 ĐK	100% (2)					100% (2)
Bình Giai	0 (4)	40.3% (154)				39.2% (158)
Bình Hà 1	100% (1)	42.4% (85)				43.0% (86)
Bình Hà 2		43.1% (116)				43.1% (116)
Bình Tân	30.0% (10)					30.0% (10)
Bình Thủy	50.0% (4)					50.0% (4)
Bình Tiến	16.7% (6)		100% (1)		100% (1)	37.5% (8)
Bù Tam		7.1% (14)	35.7% (14)	38.2% (34)		30.6% (62)
Total	53.0% (134)	44.5% (1295)	41.9% (31)	44.2% (43)	50.0% (8)	45.2% (1511)

Table 13. Percentage of smear positive individuals harbouring vivax by hamlet and ethnic group. Figures in brackets are total number of smear positive observations in the hamlet-ethnic group subsample

		Pf parasitaemia
Any symptom	Symptomatic	2000 (400-7600)
	Presymptomatic	1200 (600-2000)
	Asymptomatic	1800 (200-5600)
	Total	1400 (400-6600)
Excluding headache alone	Symptomatic	3000 (400-8400)
	Presymptomatic	1200 (600-2200)
	Asymptomatic	800 (200-4200)
	Total	1400 (400-6600)

Table 14. P.falciparum parasitaemia (median (interquartile range)) and symptom status

Genotype	P.falciparum	P.vivax	P.malariae	Mixed	Total
AA	54.5%	32.7%	0.7%	12.1%	774
AE	56.2%	28.5%	1.9%	13.3%	473
EE	56.4%	27.6%	1.3%	14.7%	156
Total	776	431	16	180	1403

Table 15. Species distribution by genotype

Age group	Vivax		
	AA	AE	EE
<1	5.4% (37)	0 (30)	0 (8)
1	7.5% (40)	5.2% (58)	0 (11)
2-4	14.8% (209)	15.6% (154)	15.9% (44)
5-9	16.6% (301)	16.1% (261)	17.9% (67)
10-14	10.0% (160)	16.3% (153)	5.3% (57)
15-19	10.0% (110)	7.7% (104)	18.9% (53)
20-29	4.3% (230)	6.4% (219)	6.2% (65)
30-39	3.9% (179)	3.6% (166)	0 (37)
40-49	9.5% (105)	5.7% (105)	5.9% (34)
50-59	4.2% (96)	1.0% (99)	3.6% (28)
60-69	1.5% (67)	2.6% (77)	5.6% (18)
70+	0 (30)	0 (28)	0 (16)

Age group	Vivax alone		
	AA	AE	EE
<1	50.0% (2)	0 (1)	0 (0)
1	100.0% (3)	30.0% (10)	0 (0)
2-4	46.0% (50)	34.1% (44)	50.0% (12)
5-9	39.4% (99)	32.9% (79)	30.0% (20)
10-14	30.8% (39)	35.2% (54)	11.1% (18)
15-19	32.1% (28)	25.0% (28)	35.3% (17)
20-29	24.2% (33)	26.2% (42)	36.4% (11)
30-39	28.6% (21)	29.4% (17)	0 (3)
40-49	56.3% (16)	23.5% (17)	14.3% (7)
50-59	40.0% (10)	0 (5)	50.0% (2)
60-69	0 (4)	20.0% (5)	0 (2)
70+	0 (3)	0 (4)	0 (0)

Age group	Falciparum		
	AA	AE	EE
<1	2.7% (37)	3.3% (30)	0 (8)
1	0 (40)	12.1% (58)	0 (11)
2-4	12.9% (209)	18.2% (154)	13.6% (44)
5-9	19.6% (301)	20.3% (261)	20.9% (67)
10-14	16.9% (160)	20.9% (153)	28.1% (57)
15-19	17.3% (110)	18.3% (104)	20.8% (53)
20-29	10.9% (230)	13.2% (219)	10.8% (65)
30-39	7.8% (179)	7.2% (166)	8.1% (37)
40-49	6.7% (105)	12.4% (105)	17.6% (34)
50-59	6.3% (96)	5.1% (99)	3.6% (28)
60-69	6.0% (67)	5.2% (77)	5.6% (18)
70+	10.0% (30)	14.3% (28)	0 (16)

Age group	Falciparum alone		
	AA	AE	EE
<1	0 (2)	100.0% (1)	0 (0)
1	0 (3)	70.0% (10)	0 (0)
2-4	38.0% (50)	43.2% (44)	41.7% (12)
5-9	47.5% (99)	45.6% (79)	40.0% (20)
10-14	56.4% (39)	46.3% (54)	77.8% (18)
15-19	60.7% (28)	64.3% (28)	41.2% (17)
20-29	69.7% (33)	59.5% (42)	63.6% (11)
30-39	61.9% (21)	64.7% (17)	100.0% (3)
40-49	37.5% (16)	64.7% (17)	71.4% (7)
50-59	60.0% (10)	80.0% (5)	50.0% (2)
60-69	75.0% (4)	60.0% (5)	0 (2)
70+	100.0% (3)	100.0% (4)	0 (0)

Table 16. Age group and species (percentage positive (denominator)) by genotype in the S'tiêng

Ethnicity	Age group	Genotype		
		AA	AE	EE
Kinh	<2	0.8%	0	
	2-9	3.7%	12.7%	0
	10-19	5.7%	8.8%	
	20-39	5.8%	7.0%	
	40-59	4.6%	9.1%	
	60+	1.3%	0	
S'tiếng	<2	8.4%	12.5%	9.7%
	2-9	31.3%	31.9%	38.4%
	10-19	24.9%	34.9%	30.7%
	20-39	14.4%	15.4%	16.8%
	40-59	12.7%	12.2%	12.9%
	60+	9.4%	8.5%	6.3%
Tày	<2	0	0	
	2-9	1.7%	0	
	10-19	7.0%	0	
	20-39	9.8%	0	
	40-59	5.9%	0	
	60+	2.3%	0	
Nùng	<2	0	0	
	2-9	1.7%	0	
	10-19	17.4%	33.3%	
	20-39	12.6%	50.0%	
	40-59	15.5%	0	
	60+	3.8%	0	
M'Nông	<2	0	4.3%	33.3%
	2-9	20.2%	15.7%	28.6%
	10-19	15.9%	4.0%	100.0%
	20-39	15.5%	7.0%	28.6%
	40-59	6.4%	3.8%	0
	60+	0	6.7%	0
Other	<2	12.5%	0	0
	2-9	2.3%	0	33.3%
	10-19	11.1%	0	100.0%
	20-39	11.1%	18.2%	0
	40-59	0	0	
	60+	0	0	

Table 17. Percentage smear positive by age and genotype.

CASE CONTROL

	Percentage (number) of patients with severe disease using healthcare provider	Percentage (number) of patients with severe diseases not using healthcare provider
Hospital or health station	47% (32)	47% (16)
Private doctor, pharmacist or market	48% (48)	56% (5)
Traditional medicine	58% (11)	46% (19)

Table 18. Treatment modality and severity

Hospital or health station	47% (58)
Market trader, private doctor or pharmacist	56% (59)
Traditional medicine	56% (9)

Table 19. Percentage of patients receiving different types of treatment in two weeks prior to transmission with severe disease. Numbers in parentheses are total numbers of individuals using that treatment modality/healthcare provider

Syndrome	Number of cases with syndrome	Mean (SEM) age of cases with syndrome	Number of cases without syndrome	Mean (SEM) age of cases without syndrome	p value
Anaemia	47	17.7 (2.12)	263	23.8 (1.03)	0.193
Jaundice	97	28.3 (1.63)	213	20.45 (1.11)	0.0001
Cerebral	142	25.0 (1.42)	168	21.1 (1.25)	0.039
Haemoglobinuria	45	22.9 (2.25)	265	22.9 (1.03)	0.985
Bleeding	11	28.7 (4.95)	299	22.7 (0.96)	0.236
Renal failure	24	35.1 (2.96)	285	21.8 (0.97)	0.0001
Respiratory distress	30	33.6 (2.94)	280	21.75 (0.97)	0.0002
Hyperparasitaemia	106	19.2 (1.57)	204	24.8 (1.15)	0.0042
Shock	3	33 (12.8)	307	22.8 (0.94)	0.290
Acidosis	4	40.25 (5.81)	306	22.7 (0.94)	0.035
Hyperlactataemia	27	34.4 (3.47)	283	21.8 (0.95)	0.0001

Table 20: Unadjusted age – clinical syndrome associations

Syndrome	Number of cases with syndrome	Mean (SEM) age of cases with syndrome	Number of cases without syndrome	Mean (SEM) age of cases without syndrome	p value
Anaemia	41	15.4 (2.08)	191	20.2 (1.17)	0.0751
Jaundice	48	20.1 (1.97)	184	19.2 (1.20)	0.7134
Cerebral	98	20.8 (1.67)	134	18.3 (1.31)	0.2355
Haemoglobinuria	29	21.3 (2.97)	203	19.1 (1.11)	0.4973
Bleeding	6	22.8 (7.21)	226	19.3 (1.05)	0.5897
Renal failure	6	38.0 (9.33)	226	18.9 (1.02)	0.0032
Respiratory distress	11	31.5 (6.82)	221	18.8 (1.02)	0.0091
Hyperparasitaemia	88	16.9 (1.71)	144	20.9 (1.29)	0.0563
Hyperlactataemia	13	26.7 (5.20)	219	19.0 (1.05)	0.0862

Table 21: Age association of syndromes at Phước Long and Đồng Xoài combined

Syndrome	Number of cases with syndrome	Mean (SEM) age of cases with syndrome	Number of cases without syndrome	Mean (SEM) age of cases without syndrome	p value
Anaemia	6	33.3 (5.52)	72	33.3 (1.72)	1
Jaundice	49	36.2 (2.04)	29	28.4 (2.48)	0.0192
Cerebral	44	34.3 (2.08)	34	32.1 (2.61)	0.4955
Haemoglobinuria	16	26.0 (3.33)	62	35.2 (1.80)	0.0213
Bleeding	5	35.8 (5.83)	73	33.2 (1.70)	0.6948
Renal failure	18	34.2 (2.66)	59	32.8 (1.99)	0.7362
Respiratory distress	19	34.8 (2.60)	59	32.8 (1.99)	0.6027
Hyperparasitaemia	18	30.5 (2.85)	60	34.2 (1.94)	0.3444
Shock	2	45.5 (4.50)	76	33.0 (1.66)	0.2283
Acidosis	4	40.3 (5.81)	74	33.0 (1.69)	0.3271
Hyperlactataemia	14	41.6 (3.89)	64	31.5 (1.73)	0.0171

Table 22. Age association of syndromes at HTD

Syndrome	Percentage of children with syndrome	Percentage of adults with syndrome	p value
Anaemia	20.4	14.5	0.235
Jaundice	18.6	23.4	0.365
Cerebral	40.7	42.7	0.751
Haemoglobinuria	8.9	15.3	0.129
Bleeding	2.7	2.4	0.908
Renal failure	0.9	4.0	0.123
Respiratory distress	2.7	6.5	0.165
Hyperparasitaemia	46.0	30.7	0.015
Shock	0.9	0	0.294
Hyperlactataemia	3.5	7.3	0.209
Total Number	113	124	

Table 23. Syndrome categories in children and adults from Phước Long and Đồng Xoài

Syndrome	Percentage of under 10's with syndrome	Percentage of over 10's with syndrome	p value
Anaemia	22.4	14.5	0.124
Jaundice	14.1	25.0	0.049
Cerebral	42.4	41.5	0.892
Haemoglobinuria	9.4	13.8	0.321
Bleeding	1.2	3.3	0.321
Renal failure	1.2	3.3	0.321
Respiratory distress	3.5	5.3	0.543
Hyperparasitaemia	50.6	30.9	0.003
Shock	1.2	0	0.180
Hyperlactataemia	3.5	6.6	0.323
Total Number	85	152	

Table 24. Syndrome categories in younger children and older children and adults in Phước Long and Đồng Xoài

Syndrome	Percentage of Kinh with syndrome	Percentage of S'tiếng with syndrome	p value
Anaemia	12.3	25.0	0.019
Jaundice	34.9	21.2	0.055
Cerebral	48.1	38.5	0.208
Haemoglobinuria	15.7	13.5	0.679
Bleeding	3.8	0	0.152
Renal failure	8.6	1.9	0.098
Respiratory distress	10.2	3.9	0.148
Hyperparasitaemia	30.6	44.2	0.059
Shock	0.9	1.9	0.492
Acidosis	1.3	0	0.413
Hyperlactataemia	8.9	5.8	0.455
Total number	235	52	

Table 25. Syndrome categories in Kinh and S'tiếng ethnic groups (all centres)

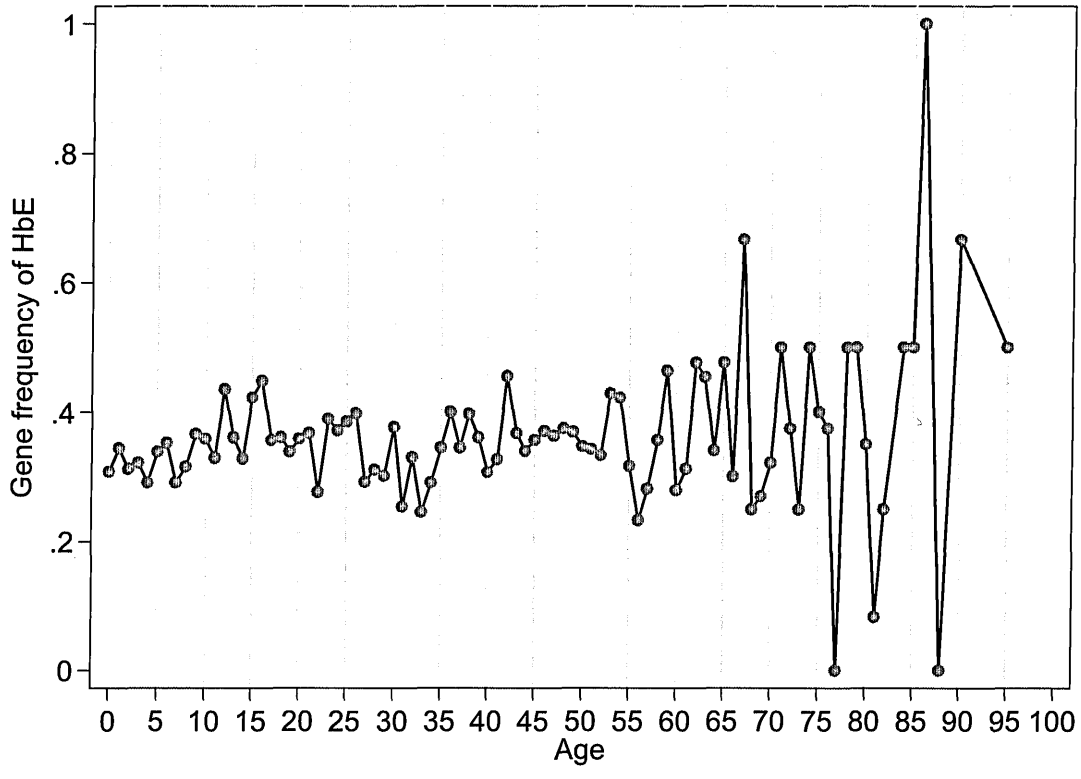


Fig 7. Relationship between prevalence of HbE and age in all surveys amongst the S'tiếng.

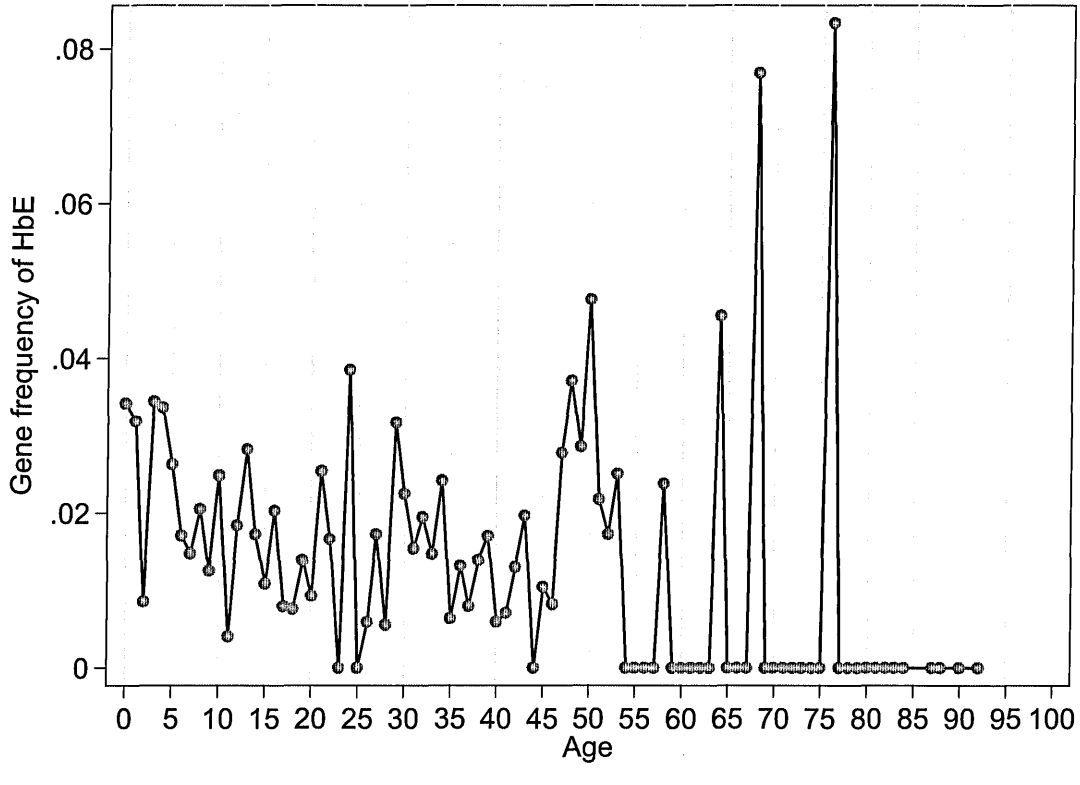


Fig 8. Relationship between prevalence of HbE and age in all surveys amongst the Kinh.

Average E gene freq in controls	Case genotype		
	AA	AE	EE
0	1	**2**	**1**
0.5	*3*	**2**	**0**
1	*2*	4	**0**
1.5	*0*	*2*	**0**
2	*2*	*0*	0

Cases with α thalassaemia

Case genotype		
AA	AE	EE
2	**3**	**1**
1	**2**	**0**
5	3	**0**
1	*1*	**0**
1	*1*	0

Cases without α thalassaemia

Tables 26 & 27. Relationships between case and control Haemoglobin E genotype for cases with (26) and without (27) coinherited α thalassaemia. Controls are included regardless of α thalassaemia status.

		Case genotype (number of intact α globin genes)			
		1	2	3	4
Average number of intact α globin genes amongst controls	2	**0**	0	*1*	*0*
	2.5	**0**	**0**	*0*	*2*
	3	**0**	**0**	3	*4*
	3.5	**0**	**1**	**3**	*6*
	4	**1**	**0**	**2**	4

Cases with Haemoglobin E

Case genotype (number of intact α globin genes)			
1	2	3	4
0	0	*0*	*0*
0	**0**	*0*	*1*
1	**2**	2	*3*
0	**0**	**1**	*3*
0	**1**	**0**	3

Cases without Haemoglobin E

Tables 28 & 29. Relationships between case and control α thalassaemia genotype for cases with (28) and without (29) coinherited Haemoglobin E. Controls are included regardless of α thalassaemia status.

Control 1 genotype	Control 2 genotype			
	AA	AE	EE	Total
AA	147 (97%, 97%)	4 (3%, 80%)	1 (1%, 100%)	152 (96%)
AE	5 (71%, 3%)	1 (14%, 20%)	0	6 (4%)
EE	0	0	0	0
Total	152 (96%)	5 (3%)	1 (1%)	158

Table 30. Comparison of HbE genotypes between pairs of Kinh controls. Cell contents are number (row percentage, column percentage) with the meaningless percentage omitted from totals columns.

Control 1 genotype	Control 2 genotype			
	AA	AE	EE	Total
AA	10 (45%, 48%)	8 (36%, 47%)	4 (18%, 40%)	22 (45%)
AE	7 (44%, 33%)	7 (44%, 41%)	2 (13%, 20%)	16 (33%)
EE	4 (40%, 19%)	2 (20%, 12%)	4 (40%, 40%)	10 (21%)
Total	21 (44%)	17 (35%	10 (21%)	48

Table 31. Comparison of HbE genotypes between pairs of S'tiêng controls. Cell contents are number (row percentage, column percentage) with the meaningless percentage omitted from totals columns.

Comparator	Ethnic group	Any HbE	Heterozygous HbE	Homozygous HbE
All matched controls	All	1.1 (0.72)	1.4 (0.28)	0.3 (0.07)
	S'tiêng	1.5 (0.29)	2.1 (0.08)	0.3 (0.08)
First matched control	All	1.1 (0.74)	1.4 (0.40)	0.4 (0.14)
	S'tiêng	1.6 (0.25)	2.4 (0.07)	0.3 (0.15)

Table 32. Comparison of conditional logistic regression analyses performed with one and both community controls. Cell contents are odds ratios (p values).

KAP

Ethnic category	KAP	Population	p
S'tiêng	6.0+/-0.14	5.5+/-0.09	<0.001
Not S'tiêng	4.6+/-0.10	4.5+/-0.07	0.336
Mixed	5.2+/-0.80	5.5+/-0.09	0.766
Total	5.3+/-0.09	5.0+/-0.06	0.006

Table 33. Comparison of family size (mean number of household members+/-SE) in KAP sample and census data, by ethnic category.

Ethnic category	KAP	Population	p
S'tiêng	51.8% (1550)	52.1% (3447)	0.854
Not S'tiêng	46.1% (1268)	46.7% (2778)	0.734
Total	49.2% (2818)	49.7% (6225)	0.683

Table 34. Comparison of percentages of women and girls (total sample size) in KAP and census data, by ethnic category.

Ethnic category	KAP	Population	p
S'tiêng	22.4+/-0.47	23.3+/-0.32	0.0961
Not S'tiêng	22.9+/-0.46	23.4+/-0.32	0.3505
Total	22.6+/-0.33	23.4+/-0.23	0.0605

Table 35. Comparison of mean age (+/- SE) of KAP sample with census data by ethnic category.

Age group	Not S'tiêng		S'tiêng		Total	
	Population	KAP	Population	KAP	Population	KAP
<2	**2.6%**	**4.3%**	**2.2%**	**4.5%**	**2.4%**	**4.4%**
2-4	**10.6%**	**8.2%**	11.4%	9.9%	**11.0%**	**9.2%**
5-9	13.2%	14.3%	**15.5%**	**18.0%**	**14.5%**	**16.3%**
10-14	10.8%	11.3%	12.9%	12.5%	11.9%	12.0%
15-24	20.5%	19.8%	20.6%	18.5%	20.6%	19.1%
25-34	18.7%	18.2%	12.8%	12.4%	15.4%	15.0%
35-44	11.5%	12.7%	9.8%	9.7%	10.6%	11.0%
45-54	6.7%	8.0%	7.1%	6.6%	6.9%	7.2%
55-64	**3.7%**	**2.3%**	5.7%	4.7%	**4.8%**	**3.6%**
65+	1.7%	1.1%	**2.1%**	**3.2%**	1.9%	2.2%
Total	2,755	1,269	3,403	1,550	6,158	2,819

Table 36. Comparison of age group structure between KAP and census data, by ethnic category. Bold indicates statistically significant difference between proportions in census and KAP.

Respondent	Number of households
Head of household	407
Husband or wife	109
Adult offspring	31
Mother	5
Son-in-law	3
Daughter-in-law	3
Sister or brother in law	2
Sibling	2
Not recorded	37

Table 37. Relationship of interviewee for household questionnaire to head of household.

Respondent	Kinh	S'tiêng	Tày	Nùng	Hoa	Mixed	Total
HoH	104 (78%)	173 (73%)	20 (69%)	21 (68%)	14 (56%)	21 (51%)	356 (71%)
Wife	28 (21%)	33 (14%)	8 (28%)	9 (29%)	7 (28%)	12 (29%)	99 (20%)
Child	2 (1%)	20 (8%)	0	1 (3%)	2 (8%)	3 (7%)	28 (6%)
Mother	0	2 (1%)	1 (3%)	0	1 (4%)	1 (2%)	5 (1%)
Daughter-in-law	0	3 (1%)	0	0	0	0	3 (1%)
Husband	0	1 (0%)	0	0	0	2 (5%)	3 (1%)
Son-in-law	0	2 (1%)	0	0	0	1 (2%)	3 (1%)
Sibling	0	1 (0%)	0	0	1 (4%)	0	2 (0%)
Sister or brother in law	0	1 (0%)	0	0	0	1 (2%)	2 (0%)
Total	134	236	29	31	25	41	501

Table 38. Relationship of respondent to head of household by ethnic group.(5 families of other ethnic group not shown)

Hamlet	Total number	No nearby stream/ no data	>500m	201-500m	51-200m	<=50m
Thôn 4	74	54%	0	0	12%	34%
Thôn 7	54	56%	0	0	11%	33%
Bù Bưng	72	56%	0	0	14%	31%
Đak Khâu	81	0	7%	28%	49%	15%
Bình Giai	80	60%	25%	6%	6%	3%
Bù Tam	78	74%	0	0	24%	1%
Bù Gia Phúc 1	43	100%	0	0	0	0
Bù Gia Phúc 2	37	78%	0	0	5%	16%
Thác Dài	80	9%	3%	26%	51%	11%
Total	599	49%	5%	8%	22%	16%

Table 39. Distances to stream by hamlet

	Thôn 4	Thôn 7	Bù Bưng	Đak Khâu	Bình Giai	Bù Tam	Bù Gia Phúc 1	Bù Gia Phúc 2	Thác Dài	Total
Kinh	12%	21%	13%	10%	9%	11%	13%	6%	5%	146
S'tiếng	12%	2%	18%	12%	8%	8%	10%	10%	19%	249
Tày	31%	24%	0	3%	17%	24%	0	0	0	29
Nùng	19%	3%	0	0	3%	75%	0	0	0	36
Hoa	0	0	0	0	100%	0	0	0	0	25
Mixed	20%	20%	20%	2%	11%	11%	0	2%	11%	44
Other	20%	0	0	0	80%	0	0	0	0	5
Total	14%	10%	13%	9%	14%	14%	8%	7%	11%	534

Table 40. Breakdown of ethnic group samples by hamlet

Unwell family member is:	Health station	Hospital	Private doctor	Depends on illness	Other
Adult	149 (25%)	93 (16%)	143 (24%)	184 (31%)	22 (4%)
Child	157 (28%)	88 (16%)	129 (23%)	166 (30%)	17 (3%)

Table 41. Comparison between household behaviour when adult is sick and that when a child is unwell.

Ethnic group	Know the cause of malaria	Think malaria is preventable	
		No	Yes
Kinh	No	47	9
	Yes	4	166
S'Tieng	No	216	10
	Yes	19	89
Tay	No	22	2
	Yes	1	17
Nung	No	41	0
	Yes	1	11
Hoa	No	7	1
	Yes	0	32
Other	No	0	0
	Yes	0	12
All	No	333	22
	Yes	25	327

Table 42. Congruence of malaria knowledge: knowledge of cause vs considering malaria preventable

Ethnic group	Know the cause of malaria	Think antimalarials are available free of charge	
		No	Yes
Kinh	No	37	15
	Yes	34	122
S'Tieng	No	111	65
	Yes	28	56
Tay	No	15	8
	Yes	7	11
Nung	No	40	1
	Yes	2	10
Hoa	No	6	0
	Yes	27	5
Other	No	0	0
	Yes	11	1
All	No	209	89
	Yes	109	205

Table 43. Congruence of malaria knowledge: knowledge of free provision of antimalarials vs considering malaria preventable

Ethnic group	Think malaria is preventable	Think antimalarials are available free of charge	
		No	Yes
Kinh	No	36	12
	Yes	36	125
S'Tieng	No	106	64
	Yes	24	57
Tay	No	15	6
	Yes	7	12
Nung	No	41	1
	Yes	1	10
Hoa	No	5	0
	Yes	28	5
Other	No	0	0
	Yes	11	1
All	No	203	83
	Yes	107	210

Table 44. Congruence of malaria knowledge: knowledge of cause vs knowledge of free provision of antimalarials.

		SickAction	Kinh	S'Tieng	Tay	Nung	Hoa	Mixed	Other
Đắc Ơ	Thôn 4	Health station	90%	100%	80%	100%		100%	0
		Hospital	0	0	0	0		0	0
		Private doctor	10%	0	20%	0		0	0
		Other	0	0	0	0		0	100%
		Total number	10	10	5	3		2	1
	Thôn 7	Health station	85%	100%	100%	100%		100%	
		Hospital	8%	0	0	0		0	
		Private doctor	8%	0	0	0		0	
		Other	0	0	0	0		0	
		Total number	13	4	2	1		5	
	Bù Bung	Health station	88%	88%				83%	
		Hospital	0	0				0	
		Private doctor	13%	6%				17%	
		Other	0	6%				0	
		Total number	8	17				6	
Đức Hạnh	Đak Khâu	Health station	0	17%	0				
		Hospital	64%	55%	100%				
		Private doctor	36%	28%	0				
		Other	0	0	0				
		Total number	14	29	1				
	Bù Gia Phúc 1	Health station	0	14%					
		Hospital	33%	71%					
		Private doctor	67%	14%					
		Other	0	0					
		Total number	6	7					
	Bù Gia Phúc 2	Health station	43%	60%					
		Hospital	14%	32%					
		Private doctor	29%	8%					
		Other	14%	0					
		Total number	7	25					
	Thác Dài	Health station	20%	18%				50%	
		Hospital	60%	47%				50%	
		Private doctor	20%	32%				0	
		Other	0	3%				0	
		Total number	5	0				0	
Đa Kia	Bình Giai	Health station	20%	19%	50%	0	30%	33%	20%
		Hospital	0	0	0	0	22%	0	0
		Private doctor	80%	81%	50%	100%	48%	67%	80%
		Other	0	0	0	0	0	0	0
		Total number	10	16	4	1	23	3	5
	Bù Tam	Health station	0	20%	17%	4%	0	25%	
		Hospital	0	0	0	0	0	0	
		Private doctor	86%	15%	67%	92%	0	75%	
		Other	14%	65%	17%	4%	0	0	
		Total number	14	20	6	25	0	4	

Table 45. Ethnic variation in health care seeking behaviour, by hamlet.

Age group	Smear positive	Total smears	p value
<2	0	16	0.047
2-4	4 (6.5%)	62	0.115
5-9	7 (7.9%)	89	0.006
10-14	1 (2.0%)	51	0.627
15-24	2 (2.2%)	92	0.585
25-34	6 (3.3%)	181	0.855
35-44	4 (2.5%)	162	0.603
45-54	0	106	0.049
55-64	1 (2.4%)	42	0.908
65+	1 (2.8%)	36	0.781
Total	26 (3.1%)	837	

Smear	Number	Mean age	p
Negative	811	30.4+/-0.65	0.032
Positive	26	22.5+/-3.72	

Table 46 Age group and mean age and smear positive prevalence in KAP.

Village	Hamlet	Smear positive (total observations)	Village smear positive
Đa Kia	Bình Giai	3% (166)	2% (334)
	Bù Tam	2% (168)	
Đức Hạnh	Bù Gia Phúc 1	4% (84)	4% (503)
	Bù Gia Phúc 2	7% (71)	
	Đak Khâu	1% (185)	
	Thác Dài	6% (163)	
Total		3% (837)	

Table 47. Smear positivity by hamlet in KAP survey. No significant differences.

Number of nets	Bednets per person		Bednets per adult		Good bednets per person		Good bednets per adult	
	KAP	All	KAP	All	KAP	All	KAP	All
0	0 (4)	0 (4)	0 (4)	0 (4)	8% (85)	18% (112)	8% (85)	18% (112)
<0.5	8% (164)	29% (203)	4% (24)	20% (25)	7% (124)	32% (152)	9% (23)	25% (28)
1	5% (222)	19% (279)	6% (312)	22% (387)	4% (178)	19% (219)	5% (238)	22% (287)
2	0 (4)	0 (5)	8% (52)	25% (71)	0 (4)	0 (5)	7% (43)	29% (58)
3	(0)	(0)	0 (2)	67% (3)	(0)	(0)	0 (2)	50% (2)
Total	6% (394)	22% (491)	6% (394)	22% (491)	6% (391)	22% (488)	6% (391)	22% (488)

Table 48. Smear positivity by number and state of bednets. Number of bednets per person rounded to nearest integer, but households with >0 but <0.5 bednets per denominator tabulated separately from those with no bednets or no good bednets. Cell contents are percentage of households smear positive (total number of households). No significant differences when examined as continuous variables.

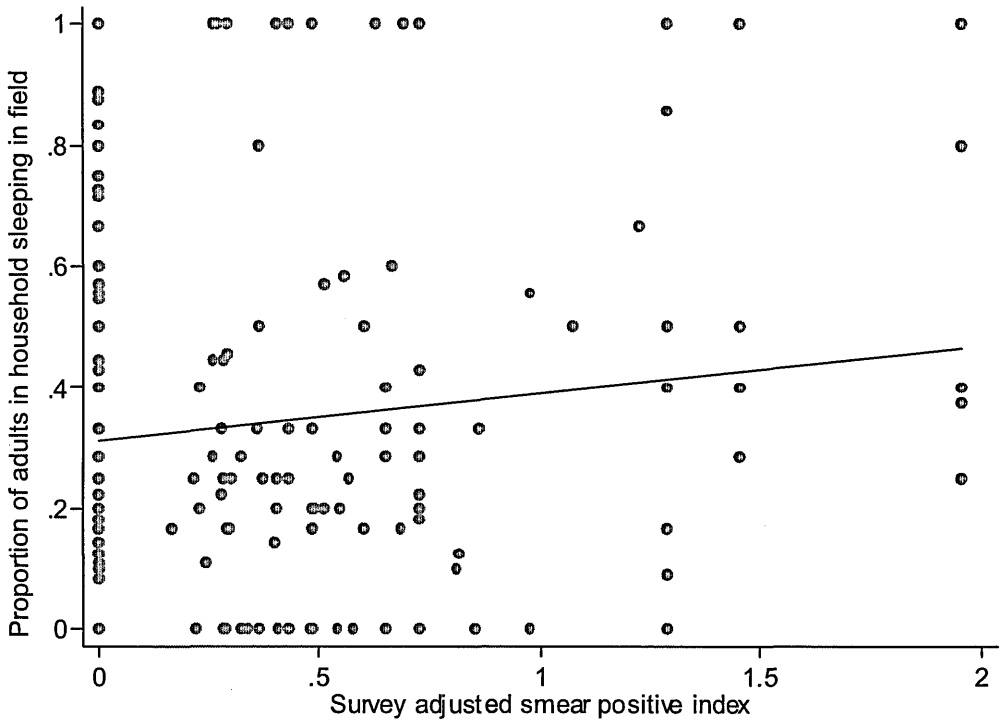


Fig 10. Relationship between proportion of adults in a house who admit to sleeping in the field and the survey adjusted household smear positive index. Linear regression p=0.06.

	Distance to stream (Mean+/-SE)			Stream within 250m (% smear positive (total number))			Stream within 500m (% smear positive (total number))		
Survey	Smear negative	Smear positive	p	Yes	No	p	Yes	No	p
KAP	418+/-55	411+/-207	0.979	3.7% (137)	5.33% (75)	0.561	3.5% (172)	7.5% (40)	0.257
All	344+/-48	361+/-105	0.875	17.0% (229)	16.0% (75)	0.836	17.4% (264)	12.5% (40)	0.437
				(Mean+/-SE)		p	(Mean+/-SE)		p
Mean adjusted smear index				0.08+/-0.027	0.045+/-0.0086	0.098	0.056+/-0.011	0.05+/-0.017	0.798

Table 49. Distance to stream and household malaria risk

Survey	Smear positive	Smear negative	p
KAP	18+/-8.3	13+/-1.4	0.394
All	10+/-2.2	14+/-1.4	0.130

Table 50. Distance from house to road by house smear status

	Survey	KAP					All surveys				
		Families without crop		Families with crop		p	Families without crop		Families with crop		p
Crop	Ethnic group	N	Percent positive	N	Percent positive		N	Percent positive	N	Percent positive	
Cashew	Kinh	21	10%	60	3%	0.26	22	14%	75	8%	0.423
	S'tiếng	36	6%	132	8%	0.58	39	18%	181	34%	0.047
	Tày	4	0	9	0	N/A	4	25%	18	17%	0.696
	Nùng	6	0	22	9%	0.443	6	0	29	24%	0.178
	Hoa	13	15%	12	0	0.157	13	15%	12	0	0.157
	Total	99	6%	298	6%	0.994	105	13%	389	25%	0.013
Pepper	Kinh	54	6%	27	4%	0.717	62	13%	35	3%	0.101
	S'tiếng	164	8%	4	0	0.558	202	32%	18	28%	0.732
	Tày	9	0	4	0	N/A	14	29%	8	0	0.095
	Nùng	10	20%	18	0	0.049	13	31%	22	14%	0.221
	Hoa	15	13%	10	0	0.229	15	13%	10	0	0.229
	Total	321	7%	76	1%	0.054	381	26%	113	10%	<0.001
Rice	Kinh	77	5%	4	0	0.64	92	10%	5	0	0.463
	S'tiếng	85	9%	83	6%	0.411	87	24%	133	36%	0.062
	Tày	7	0	6	0	N/A	8	25%	14	14%	0.531
	Nùng	3	0	25	8%	0.611	5	0	30	23%	0.227
	Hoa	25	8%	0	N/A	N/A	25	8%	0	0	N/A
	Total	255	6%	142	6%	0.797	283	16%	211	31%	<0.001
Cassava	Kinh	61	5%	20	5%	0.988	73	10%	24	8%	0.854
	S'tiếng	137	9%	31	0	0.074	179	32%	41	29%	0.749
	Tày	4	0	9	0	N/A	10	20%	12	17%	0.84
	Nùng	1	0	27	7%	0.778	3	33%	32	19%	0.546
	Hoa	25	8%	0	N/A	N/A	25	8%	0	0	N/A
	Total	293	7%	104	3%	0.115	365	23%	129	19%	0.359
Coffee	Kinh	66	5%	15	7%	0.732	74	9%	23	9%	0.912
	S'tiếng	167	8%	1	0	0.771	218	31%	2	50%	0.568
	Tày	11	0	2	0	N/A	19	21%	3	0	0.38
	Nùng	27	7%	1	0	0.778	34	21%	1	0	0.612
	Hoa	0	N/A	25	8%	N/A	0	N/A	25	8%	N/A
	Total	335	6%	62	5%	0.664	418	25%	76	8%	0.001

Table 51. Smear positivity by crop choice and ethnic group in the KAP and all surveys.

		KAP						All surveys					
Healthcare provider chosen	Ethnicity	Health station	Hospital	Private doctor	Depends on illness	Other	Total	Health station	Hospital	Private doctor	Depends on illness	Other	Total
last time adult in household was ill	Kinh	17% (6)	0 (15)	9% (32)	0 (24)	0 (3)	5% (80)	17% (12)	0 (15)	**19% (32)**	**0 (34)**	33% (3)	9% (96)
	S'tiếng	6% (34)	4% (47)	10% (39)	13% (32)	0 (14)	7% (166)	36% (53)	30% (47)	30% (40)	36% (64)	**0 (14)**	31% (218)
	Tày	0 (3)	0 (1)	0 (6)	0 (2)	0 (1)	0 (13)	**43% (7)**	0 (1)	0 (6)	14% (7)	0 (1)	18% (22)
	Nùng	0 (1)	N/A	4% (24)	0 (2)	100% (1)	7% (28)	50% (4)	N/A	13% (24)	17% (6)	100% (1)	20% (35)
	Hoa	29% (7)	0 (5)	0 (11)	0 (1)	N/A	8% (24)	**29% (7)**	0 (5)	0 (11)	0 (1)	N/A	8% (24)
	Mixed	0 (3)	0 (1)	0 (5)	0 (3)	N/A	0 (12)	13% (8)	0 (1)	0 (5)	22% (9)	N/A	13% (23)
	Other	0 (1)	N/A	0 (4)	N/A	N/A	0 (5)	0 (1)	N/A	0 (4)	N/A	100% (1)	17% (6)
	Total	9% (55)	3% (69)	7% (121)	6% (64)	5% (19)	6% (328)	32% (92)	20% (69)	17% (122)	22% (121)	15% (20)	22% (424)
last time child in household was ill	Kinh	29% (7)	0 (15)	7% (29)	0 (24)	0 (2)	5% (77)	**31% (13)**	0 (15)	14% (29)	**0 (34)**	0 (2)	9% (93)
	S'tiếng	9% (35)	2% (44)	14% (36)	9% (32)	0 (11)	8% (158)	38% (56)	27% (44)	30% (37)	36% (61)	**0 (11)**	32% (209)
	Tày	0 (3)	0 (1)	0 (7)	0 (1)	0 (1)	0 (13)	**43% (7)**	0 (1)	14% (7)	0 (6)	0 (1)	18% (22)
	Nùng	0 (1)	100% (1)	5% (22)	0 (2)	N/A	8% (26)	40% (5)	100% (1)	14% (22)	20% (5)	N/A	21% (33)
	Hoa	29% (7)	0 (4)	0 (11)	0 (1)	N/A	9% (23)	29% (7)	0 (4)	0 (11)	0 (1)	N/A	9% (23)
	Mixed	0 (3)	0 (2)	0 (5)	0 (1)	N/A	0 (11)	25% (8)	0 (2)	0 (5)	17% (6)	N/A	14% (21)
	Other	N/A	N/A	0 (3)	N/A	N/A	0 (3)	N/A	N/A	0 (3)	N/A	100% (1)	25% (4)
	Total	13% (56)	3% (67)	7% (113)	5% (61)	0 (14)	6% (311)	35% (96)	19% (67)	17% (114)	21% (113)	7% (15)	22% (405)

Table 52. Healthcare provider choice at household level and risk of malaria. Cell contents are percentage smear positive (total number of the specified ethnic group choosing the specified healthcare provider). Bold font indicates $p<0.05$ for the specified healthcare provider against all others in the specified ethnic group. Cells that reach significance but on a very small number of observations are not presented in bold font. The number of events in the KAP study were to small to consider whether a cell might meet significance.

		KAP						All surveys					
Source of medicine last time	Ethnicity	Market	Private doctor	Health station - paid	Health station - free	Hospital	Total	Market	Private doctor	Health station - paid	Health station - free	Hospital	Total
adult in household was ill	Kinh	6% (18)	5% (41)	13% (8)	0 (1)	0 (4)	6% (72)	4% (25)	9% (43)	21% (14)	0 (1)	0 (5)	9% (88)
	S'tiếng	0 (26)	7% (68)	10% (20)	0 (7)	8% (12)	6% (133)	27% (44)	34% (71)	33% (40)	40% (15)	25% (12)	32% (182)
	Tày	0 (5)	0 (2)	N/A	0 (2)	0 (1)	0 (10)	27% (11)	0 (3)	50% (2)	0 (2)	0 (1)	21% (19)
	Nùng	5% (21)	20% (5)	N/A	0 (1)	N/A	7% (27)	17% (24)	33% (6)	33% (3)	0 (1)	N/A	21% (34)
	Hoa	N/A	0 (7)	0 (3)	N/A	N/A	0 (10)	N/A	0 (7)	0 (3)	N/A	N/A	0 (10)
	Mixed	0 (2)	0 (5)	0 (1)	0 (1)	N/A	0 (9)	29% (7)	14% (7)	0 (5)	0 (1)	N/A	15% (20)
	Other	N/A	0 (3)	0 (1)	N/A	N/A	0 (4)	100% (1)	0 (3)	0 (1)	N/A	N/A	20% (5)
	Total	3% (72)	6% (131)	9% (33)	0 (12)	6% (17)	5% (265)	21% (112)	22% (140)	26% (68)	30% (20)	17% (18)	23% (358)
child in household was ill	Kinh	5% (19)	3% (33)	0 (3)	N/A	0 (1)	4% (56)	4% (26)	6% (34)	13% (8)	N/A	0 (1)	6% (69)
	S'tiếng	6% (16)	11% (46)	6% (16)	17% (12)	0 (9)	9% (99)	26% (27)	35% (48)	34% (35)	38% (24)	**0 (10)**	31% (144)
	Tày	0 (5)	0 (3)	0 (1)	0 (1)	N/A	0 (10)	38% (8)	0 (5)	33% (3)	0 (3)	N/A	21% (19)
	Nùng	0 (19)	33% (3)	N/A	0 (1)	N/A	4% (23)	14% (22)	25% (4)	33% (3)	100% (1)	N/A	20% (30)
	Hoa	N/A	0 (3)	100% (1)	N/A	N/A	25% (4)	N/A	0 (3)	100% (1)	N/A	N/A	25% (4)
	Mixed	0 (1)	0 (5)	0 (1)	0 (1)	N/A	0 (8)	25% (4)	17% (6)	17% (6)	0 (2)	N/A	17% (18)
	Other	N/A	0 (2)	N/A	N/A	N/A	0 (2)	100% (1)	0 (2)	N/A	N/A	N/A	33% (3)
	Total	3% (60)	7% (95)	9% (22)	13% (15)	0 (10)	6% (202)	18% (88)	21% (102)	30% (56)	33% (30)	0 (11)	22% (287)

Table 53. Last source of medicine and household risk of malaria. Cell contents and formatting as for table 52.

Survey	Ethnic group	Health station	Hospital	Private office	Other/ no Rx	Self treat	Health volun-teer	Varied within household	Total
KAP	Kinh	0 (5)	7% (14)	4% (28)	0 (1)	0 (7)	N/A	12% (17)	6% (72)
	S'tiếng	7% (29)	13% (16)	7% (43)	0 (1)	0 (26)	0 (1)	17% (23)	8% (139)
	Tày	N/A	0 (1)	0 (5)	N/A	0 (2)	N/A	0 (1)	0 (9)
	Nùng	N/A	0 (1)	0 (5)	N/A	8% (12)	N/A	100% (1)	11% (19)
	Hoa	100% (1)	0 (6)	0 (7)	N/A	N/A	N/A	100% (1)	13% (15)
	Mixed	0 (2)	0 (2)	0 (2)	N/A	0 (1)	N/A	0 (2)	0 (9)
	Other	N/A	0 (2)	0 (1)	0 (1)	N/A	N/A	0 (1)	0 (5)
	Total	8% (37)	7% (42)	4% (91)	0 (3)	2% (48)	0 (1)	17% (46)	7% (268)
All surveys	Kinh	18% (11)	7% (14)	11% (28)	0 (1)	0 (10)	N/A	12% (17)	10% (81)
	S'tiếng	40% (42)	24% (17)	32% (44)	100% (1)	19% (27)	0 (1)	46% (24)	33% (156)
	Tày	25% (4)	67% (3)	0 (6)	N/A	50% (2)	N/A	0 (1)	25% (16)
	Nùng	50% (4)	0 (1)	17% (6)	N/A	15% (13)	N/A	100% (1)	24% (25)
	Hoa	100% (1)	0 (6)	0 (7)	N/A	N/A	N/A	100% (1)	13% (15)
	Mixed	14% (7)	0 (2)	0 (2)	0 (1)	50% (2)	N/A	0 (2)	13% (16)
	Other	N/A	0 (2)	0 (1)	0 (1)	100% (1)	N/A	0 (1)	17% (6)
	Total	35% (69)	16% (45)	19% (94)	25% (4)	18% (55)	0 (1)	32% (47)	24% (315)

Table 54. Healthcare provider for last malarial illness in household and risk of smear positivity. Cell contents and formatting as for table 52.

Survey	Ethnic	Self treat at home	Self treat in the field	Health station	Private doctor	Hospital	Total
KAP	Kinh	N/A	N/A	0 (8)	8% (13)	0 (6)	4% (27)
	S'tiếng	**16% (25)**	N/A	0 (18)	5% (21)	0 (5)	7% (69)
	Tày	0 (1)	N/A	0 (1)	0 (2)	0 (2)	0 (6)
	Nùng	N/A	N/A	N/A	N/A	N/A	N/A
	Hoa	N/A	0 (1)	0 (8)	0 (12)	0 (10)	0 (31)
	Other	N/A	N/A	0 (1)	0 (8)	N/A	0 (9)
	Total	15% (26)	0 (1)	0 (36)	4% (56)	0 (23)	4% (142)
All surveys	Kinh	N/A	N/A	9% (11)	15% (13)	0 (7)	10% (31)
	S'tiếng	**18% (28)**	N/A	3% (39)	8% (24)	0 (5)	8% (96)
	Tày	0 (1)	N/A	0 (2)	0 (2)	0 (2)	0 (7)
	Nùng	N/A	N/A	50% (2)	N/A	N/A	50% (2)
	Hoa	N/A	0 (1)	0 (8)	0 (12)	0 (10)	0 (31)
	Other	N/A	N/A	0 (1)	0 (8)	N/A	0 (9)
	Total	17% (29)	0 (1)	5% (63)	7% (59)	0 (24)	7% (176)

Table 55. Action on falling ill whilst in the field and individual malaria risk, by ethnic group.

Survey	Ethnic	Health station	Hospital	Private doctor	Other	Self treat	Health volunteer	Total
KAP	Kinh	0 (14)	0 (22)	2% (50)	25% (4)	0 (15)	N/A	2% (105)
	S'tiếng	3% (33)	7% (27)	4% (69)	0 (2)	2% (45)	0 (4)	4% (180)
	Tày	N/A	0 (2)	0 (8)	N/A	0 (4)	N/A	0 (14)
	Nùng	N/A	50% (2)	0 (7)	N/A	6% (17)	N/A	8% (26)
	Hoa	N/A	0 (9)	0 (6)	N/A	N/A	N/A	0 (15)
	Other	N/A	0 (4)	0 (2)	0 (1)	N/A	N/A	0 (7)
	Total	2% (47)	5% (66)	3% (142)	14% (7)	2% (81)	0 (4)	3% (347)
All surveys	Kinh	5% (20)	0 (23)	6% (51)	20% (5)	0 (17)	N/A	4% (116)
	S'tiếng	4% (51)	7% (30)	11% (74)	0 (4)	6% (47)	0 (4)	7% (210)
	Tày	0 (3)	0 (3)	0 (8)	N/A	0 (4)	N/A	0 (18)
	Nùng	33% (3)	50% (2)	13% (8)	N/A	11% (18)	N/A	16% (31)
	Hoa	N/A	0 (9)	0 (6)	N/A	N/A	N/A	0 (15)
	Other	N/A	0 (4)	0 (2)	0 (1)	N/A	N/A	0 (7)
	Total	5% (77)	4% (71)	8% (149)	10% (10)	6% (86)	0 (4)	6% (397)

Table 56. Healthcare provider for last episode of malaria and individual risk of malaria, by ethnic group.

	Survey	KAP					All surveys				
		Individuals professing not to know		Individuals professing knowledge		p	Individuals professing not to know		Individuals professing knowledge		p
Knowledge	Commune	N	Percent positive	N	Percent positive		N	Percent positive	N	Percent positive	
Know how malaria is transmitted	Đắc Ơ						32	0	26	12%	0.048
	Duc Hanh	164	2%	173	2%	0.939	176	7%	186	5%	0.311
	Da Kia	132	2%	88	1%	0.537	133	5%	90	6%	0.924
	Total	296	2%	261	2%	0.716	341	6%	302	6%	0.898
Think malaria is preventable	Đắc Ơ						32	0	26	12%	0.048
	Duc Hanh	156	3%	148	1%	0.447	169	8%	158	4%	0.091
	Da Kia	135	2%	86	1%	0.565	136	6%	88	5%	0.664
	Total	291	2%	234	1%	0.349	337	7%	272	5%	0.357
Know antimalarials are free	Đắc Ơ						8	0	50	6%	0.477
	Duc Hanh	103	4%	118	3%	0.57	111	7%	126	4%	0.274
	Da Kia	195	2%	21	0	0.508	196	5%	23	9%	0.474
	Total	298	3%	139	2%	0.744	315	6%	199	5%	0.737

Table 57. Malaria related knowledge and individual risk of smear positivity, by commune.

	Survey	KAP					All surveys				
		Individuals professing not to know		Individuals professing knowledge		p	Individuals professing not to know		Individuals professing knowledge		p
Knowledge	Ethnic group	N	Percent positive	N	Percent positive		N	Percent positive	N	Percent positive	
Know how malaria is transmitted	Kinh	40	0	94	2%	0.353	43	0	106	6%	0.111
	S'tiếng	144	3%	77	3%	0.937	182	5%	95	8%	0.348
	Tày	12	0	8	0	N/A	15	0	10	0	N/A
	Nùng	38	5%	2	0	0.739	38	16%	9	11%	0.723
	Hoa	7	0	27	0	N/A	7	0	27	0	N/A
	Total	296	2%	261	2%	0.716	341	6%	302	6%	0.898
Think malaria is preventable	Kinh	38	0	97	2%	0.373	41	0	109	6%	0.125
	S'tiếng	136	3%	57	0	0.191	174	6%	74	7%	0.898
	Tày	13	0	7	0	N/A	16	0	9	0	N/A
	Nùng	39	5%	1	0	0.816	39	15%	8	13%	0.835
	Hoa	7	0	27	0	N/A	7	0	27	0	N/A
	Total	291	2%	234	1%	0.349	337	7%	272	5%	0.357
Know antimalarials are free	Kinh	60	3%	59	0	0.157	63	5%	70	4%	0.895
	S'tiếng	101	3%	41	5%	0.576	114	5%	77	6%	0.72
	Tày	16	0	2	0	N/A	16	0	7	0	N/A
	Nùng	40	5%	0	N/A	N/A	40	15%	7	14%	0.961
	Hoa	28	0	4	0	N/A	28	0	4	0	N/A
	Total	298	3%	139	2%	0.744	315	6%	199	5%	0.737

Table 58. Malaria related knowledge and individual risk of smear positivity, by ethnic group.

Appendix 3
Laboratory recipes

Table of contents

Giemsa staining of blood smears

Thin films were fixed with methanol in the field.

Slides were flooded with a freshly prepared 10% solution of Giemsa stain.

After 30 minutes, slides were washed with tap water and air dried before examination as discussed in the text.

Phenol-chloroform method for DNA extraction from whole blood

Reagents:

Lysis buffer (0.154M NH_4Cl: 0.009M $KHCO_3$)
10% SDS solution
Proteinase K (stock solution of 10mg/ml)
Tris-saturated Phenol (pH 8), warmed to room temperature
Chloroform
3M NaAcetate
100% ethanol
70% ethanol
Tris-EDTA buffer (TE)

Method

Method is for blood volumes of up to 100µl, and is conducted in eppendorf tubes

1. Make up sample volumes to 100µl with lysis buffer.
2. Add 10µl SDS solution and 10µl Proteinase K and mix well.
3. Incubate in a water bath at 37°C overnight, or for 4 hours at 55°C.
4. Add 100µl phenol, mix and centrifuge at 13000rpm for 2-3 minutes.
5. Remove the upper phase to a fresh tube and repeat step 4
6. Remove the upper phase to a fresh tube, add 100µl chloroform mix and centrifuge at 13000rpm for 2-3 minutes.
7. Remove the upper phase to a fresh tube add 1/10 th volume sodium acetate and 2 volumes 100% ethanol.
8. Mix and allow DNA to precipitate (can leave at -20°C overnight if concentration low).
9. Centrifuge samples at 13000rpm for 15 minutes.
10. Discard the supernatant
11. Add 50µl 70% ethanol to the pellet and mix.
12. Centrifuge at 13000rpm for 2-3 minutes
13. Discard supernatant.
14. Air dry pellet for about 5 minutes. If pellet is overdried sample will be difficult to resuspend.
15. Resuspend pellet in 20µl 1xTE buffer.

Alpha thalassaemia PCR's

$-\alpha^{SEA}$ PCR

Primers

Designation	Description	Primer sequence 5'-3'
Primer F	α^{SEA}	ctc tgt gtt ctc agt att gga ggg aag gag
Primer M	$-\alpha^{SEA}$ R	ata tat ggg tct gga agt gta tcc ctc cca
Primer N	αR	tga aga gcc tgc agg acc agg tca gtg acc g

PCR reaction mix

Reagent	Initial concentration	Volume added (µl)	Final concentration
Applied biosystems bufferII includes 15mM $MgCl_2$	x10	2.5	x1
dNTP's	25mM	0.2	200µM
Primers F	10µM	2.8	1.12µM
R(M)	10µM	1.5	0.6µM
N	10µM	1.1	0.44µM
Betaine	5M	3.75	0.75M
DMSO	100%	1.25	5%
Amplitaq gold	5units/µl	0.25	1.25u/reaction
DNA			100ng
Water		make up to 25µl	

PCR running conditions

Initial step at 95°C for 15 minutes, followed by 35-40 cycles of 1 minute at 95°C, 1 minute at 65°C and 2 minutes at 72°C, followed by final extension step of 10 minutes at 72°C.

Analysis

Run PCR products on 2% agarose gel in 1xTAE. If using loading dye containing Xylene cyanol and bromphenol blue, 15% ficol separation of dyes needs to be between 6 and 10 cm.

Expected fragment sizes

	Primers	PCR fragment size bp
No deletion	α^{SEA} F vs αNR	1000
Deletion	α^{SEA} F vs α^{SEA} R	700

$-\alpha^{4.2}$ PCR

Primers

Designation	Description	primer sequence 5'-3'
Primer F	$\alpha/-\alpha^{4.2}$ F	tcc tga tct ttg aat gaa gtc cga gta ggc
Primer M	$-\alpha^{4.2}$ R	atc act gat aag tca ttt cct ggg ggt ctg
Primer N	α R	tgg ggg tgg gtg tga gga gac agg aaa gag aga

PCR reaction mix

	initial concentration	volume added µl	final concentration
Applied biosystems bufferII includes 15mM $MgCl_2$	x10	2.5	x1
dNTP's	25mM	0.2	200µM
Primers F	10µM	1.0	0.8µM
R(M)	10µM	0.75	0.6µM
N	10µM	0.25	0.2µM
Betaine	5M	3.75	0.75M
DMSO	100%	1.25	5%
Platinum taq	5units/µl	0.25	1.25u/reaction
DNA			100ng
Water		make up to 25µl	

PCR running conditions

Initial step at 95°C for 15 minutes, followed by 35-40 cycles of 1 minute at 95°C, 1 minute at 65°C and 3 minutes at 72°C, followed by final extension step of 10 minutes at 72°C.

Analysis

Run PCR products on 2% agarose gel in 1xTAE. If using loading dye containing Xylene cyanol and bromphenol blue, 15% ficol separation of dyes needs to be between 6 and 10 cm.

Expected fragment sizes

	Primers	PCR fragment size bp
$-\alpha^{4.2}$ deletion	F-N	no product
	F-R(M)	1667
No deletion	F-N	1507
	F-R(M)	no product

$-\alpha^{3.7}$ PCR

Primers

Designation	Description	primer sequence 5'-3'
Primer F	$\alpha 2/-\alpha^{3.7}$ F	aag tcc acc cct tcc ttc ctc acc
Primer M	$-\alpha^{3.7}$ R	tcc atc ccc tcc tcc cgc ccc tgc ctt ttc
Primer N	$\alpha 2$ R	atg aga gaa atg ttc tgg cac ctg cac ttg

PCR reaction mix

	Initial concentration	Volume added (µl)	Final concentration
Applied biosystems bufferII includes 15mM $MgCl_2$	x10	2.5	x1
dNTP's	25mM	0.2	200µM
Primers F	10µM	0.75	0.3µM
R(M)	10µM	0.25	0.1µM
N	10µM	0.25	0.1µM
Betaine	5M	3.75	0.75M
DMSO	100%	1.25	5%
Amplitaq gold	5units/µl	0.25	1.25u/reaction
DNA			100ng
Water		make up to 25µl	

PCR running conditions

Initial step at 95°C for 15 minutes, followed by 35-40 cycles of 1 minute at 95°C, 1 minute at 65°C and 2 minutes at 72°C, followed by final extension step of 10 minutes at 72°C.

Analysis

Run PCR products on 2% agarose gel in 1xTAE. If using loading dye containing Xylene cyanol and bromphenol blue, 15% ficol separation of dyes needs to be between 6 and 10 cm.

Expected fragment sizes

	Primers	PCR fragment size bp
$-\alpha^{3.7}$ deletion	F-N	no product
	F-R(M)	1985
No deletion	F-N	2215*
	F-R(M)	2210*
* These products are not separated by electrophoresis.		

Mismatched PCR and RFLP for determination of $Hb_{ConstantSpring}$ and Hb_{Pakse}

Strategy

Haemoglobin Pakse (TAA>TAT) and Haemoglobin Constant springs (TAA>CAA) are non-deletional α-thalassaemia mutations. Both changes occur in the termination codon of the alpha 2 gene and result in the disruption of the same *Mse I* site. Since this is the case distinguishing between these two point mutations is not possible by simple PCR-RFLP methods. A mismatch PCR followed by RFLP has been developed and is available (Haematologica 2002; 87:117-125). Primers were designed to incorporate a change that when found in association with the point mutation a recognition site for a restriction endonuclease was introduced.

$Hb_{ConstantSpring}$

tga agc acc gtg ctg acc tcc aaa tac cgt taa gct g	Normal sequence
tga agc acc gtg ctg acc tcc aaa tac cgt caa gct g	Hb_{CS} sequence
acc gtg ctg acc tcc aaa taa cgt ⇒	Mismatch Primer

aaa taa cgt taa gct	Sequence across the breakpoint normal allele
ttt att gca att cgt	*Acl I* site introduced
aaa taa cgt caa gct	Sequence across the breakpoint Hb Cs allele
ttt att gca gtt cgt	No *Acl I* site introduced

Hb_{Pakse}

att cga cct cgg agc cat cgg caa	Normal sequence
ata cga cct cgg agc cat cgg caa	Hb Pakse sequence
⇐ cga act cgg agc cat cgg caa	Mismatch Primer

taa gct tgag	Sequence across the break point normal allele
att cga act c	*Hind III* site introduced
taa gct tgag	Sequence across the break point Hb Pakse allele
att cga act c	No *Hind III* site introduced

PCR reaction mix

Constituent	Initial concentration	Reaction mix volumes (µl)	Final concentration
Abgene buffer IV	10x	5	1x
$MgCl_2$	25mM	3	1.5mM
dNTP's	25mM	0.4	200µM
Betaine	5M	7.5	0.75M
DMSO	100%	2.5	5.0%
Primers F	10µM	2.5	0.5µM
R	10µM	2.5	0.5µM
Platinum taq	5u/µl	0.2	1.0unit
Water		Make up to 50	
DNA			

PCR running conditions

Initial step at 95°C for 15 minutes, followed by 30 cycles of 1 minute at 95°C, 1 minute at 60°C and 1 minutes at 72°C, followed by final extension step of 10 minutes at 72°C.

Primers

Primer	Primer sequence 5' to 3'
Hb_{CS} Forward	acc gtg ctg acc tcc aaa taa cgt
Mismatched Hb_{CS} reverse	ctc tca gga cag ggg atg gtt cag
Hb_{Pakse} reverse	aac ggc tac cga ggc tca agc
Mismatched Hb_{Pakse} forward	ccc gcc cgg acc cac a

Analysis

Digest 10-15µl PCR product in a total volume of 20µl using 1/10th total volume digestion buffer and 1-2 units of appropriate enzyme. Analyse products on 1.5% metaphor agarose gel in 1x TBE.

PCR	Digestion products Normal allele	Digestion products Mutant allele
Hb_{CS}	302	323
Hb_{Pakse}	299 256	321 256

Assays of G6PD activity

G6PD deficiency rapid test

Three reagents were prepared:

Gel: DEAE-Sephadex A50 equilibrated in 0.1M Tris- HCl with 10mM $MgCl_2$ at pH 6.5
Substrate: 5mM glucose-6-phosphate, 0.4mM $NADP^+$. 0.2% Saponin in water
Indicator: MTT (3(4,5 dimethylthiazolyl 1-2)2,5 diphenyltetrazolium bromide) and PMS (phenazine methosulphate) both at 0.025% in water

200µl of each reagent were combined in a 1ml eppendorf with a further 200µl H2O and 5-10µl of fresh blood. After mixing, the eppendorf was left to stand for 45-60 minutes, out of direct sunlight, before the degree of reduction of the dye (and hence the activity of G6PD) was assessed by the extent of blue colouration of the gel phase.

Methylene Blue decolourisation test

Materials

Solution of 0.25M dextrose and 0.2M sodium nitrite
Solution of 0.4M methylene blue chloride
Water bath at 37+/-1°C
Test blood in anticoagulant

Conduct of test

Each test consists of three tubes:

1) Control 1, containing 2ml of test blood only
2) Control 2, containing 2ml of test blood and 0.1ml of dextrose/sodium nitrite mixture
3) Test tube containing 2ml of blood, 0.1 ml of dextrose/sodium nitrite mixture and 0.1ml methylene blue solution

All 3 tubes are incubated at 37°C for 3 hours. After incubation, 0.1ml is removed from each tube and mixed with 10ml of distilled water.

Reading test results

The Test tube is then compared with Control 2. G6PD replete subjects will have decolourised the methylene blue, and the Test tube will appear the same colour as Control 2. The Test tube in completely deficient subjects will remain a deep blue, whilst partially deficient subjects will have colour change between the deficient subjects and Control 2.